Blue Copper Proteins: Gene Synthesis and Expression

Thesis by

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To Brent

and

My Parents

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Abstract

A new strategy has been developed to facilitate the total synthesis of genes. The approach involves the synthesis of segments of the gene which are cloned into a vector for amplification and proof-reading. The construction of the gene proceeds from both ends toward the middle. In the synthesis, a segment of the gene is cloned into the vector and amplified; the resulting vector now contains a newly cloned segment which importantly includes unique internal restriction sites. The vector can be opened at these sites to allow insertion of an additional segment of the structural gene. A combination of three procedures: opening, cloning, amplification, constitutes a "step" of the synthesis, and a sequence of such "steps" leads to the synthesis of the total gene.

Many unique restriction sites have been designed into the gene thusly synthesized, as their presence will greatly facilitate the use of cassette mutagenesis in subsequent structure/function studies of the protein encoded by the synthetic gene.

This synthetic strategy has several attractive features. In principle it can be used for the construction of genes of essentially any length and, allows proof-reading and correction of errors at intermediate stages in synthesis. This approach thereby avoids some of the problems inherent in the more traditional strategy in which many segments of synthetic DNA are annealed, ligated together and inserted into a vector, followed by a hoped for isolation from the many species inevitably present in the resulting mixture of synthetic genetic material of a clone containing DNA with the desired sequence.

This stepwise approach has been applied to the synthesis of a gene for the blue copper protein, plastocyanin, of poplar leaf. The synthetic gene has

been expressed in a fusion with a region of protein A under control of the λ_R promoter.

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List of Abbreviations

A	adenine
Å	angstrom
α-^{32}P-dNTP	α-^{32}P-2′-deoxynucleotide 5′-triphosphate
ATP	adenosine 5′-triphosphate
bp	base pairs
BØB	bromophenol blue
BSA	bovine serum albumin
C	cytosine
CAP	calf alkaline phosphatase
Ci	curie
cm	centimeter
DCA	dichloroacetic acid
DEAE	diethylaminoethyl
DIPEA	diisopropylethyl amine
DMAP	dimethylamino pyridine
DMSO	dimethyl sulfoxide
DMT	4,4′-dimethoxytrityl
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleotide 5′-triphosphate
DTT	dithiothreitol
ε	extinction coefficient
E. coli	*Escherichia coli*
EDTA	ethylenediaminie-tetraacetic acid
EPR	electron paramagnetic resonance spectroscopy
G	guanine
g	grams

γ-^{32}P-ATP	γ-^{32}P-adenosine 5′-triphosphate
HPLC	high performance liquid chromatography
IR	infrared absorption spectroscopy
Kd	kilodaltons
IgG	immunoglobulin of class G
IPTG	isopropyl-β-D-thioagalactoside
L	liter
M	moles/liter
µg	microgram
min	minute
µl	microliter
µM	micromolar
µm	micrometer
µmole	micromoles
mamps	milliamperes
mg	milligram
ml	milliliters
mM	millimolar
mm	millimeter
mR/h	milliroentgen per hour
mV	millivolt
mRNA	messenger RNA
MWCO	molecular weight cut-off
NaOAc	sodium acetate
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
O.D.	optical density

pmole	picomole
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
T	thymine
TAE	Tris-acetate electrophoresis buffer
TBE	Tris-borate, EDTA electrophoresis buffer
TEAAc	triethylammonium acetate
TLC	thin layer chromatography
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet
UV-vis	ultraviolet-visible absorption spectroscopy
V	volt
v/v	volume to volume
w/v	weight to volume

Chapter 1

Total Synthesis of a Gene for the Blue Copper Protein Plastocyanin

Introduction

Proteins act in biological systems in many ways; as catalysts, transport agents, hormones, cell surface receptors, electron carriers, antibodies and markers of individuality. In general, the study of protein function focuses on a protein that occurs naturally though some modifications of the native proteins are possible through chemical means or oligonucleotide directed mutagenesis. The molecular basis of function could be studied in much greater detail if one could vary at will the protein structure in order to test predictions that result from a hypothetical model of its action. These insights would also provide a rational basis for designing specific proteins, with novel and useful properties not previously available. For these reasons, the ability to generate a protein with any desired structure represents an important goal.

The amino acid sequence of a protein uniquely determines its three-dimensional structure and therefore its function. The amino acid sequence, is in turn, determined by the sequence of bases in the DNA of the structural gene that encode the protein. Methods for efficient oligonucleotide synthesis have been recently developed which, along with other techniques of molecular biology, now allow the creation of a protein with any desired amino acid sequence. This process involves preparation of the appropriate gene sequence, either by total synthesis or specific mutation of a naturally occurring gene, followed by expression of this gene in an appropriate microbiological host.

The protein considered in this thesis is the blue copper protein, plastocyanin, which is responsible for essential redox processes in many plants (involving one electron oxidation-reduction of the copper). The objective of this project is to prepare a system which will allow the preparation of structural variants of plastocyanin in order to study the origin of the particular, and unusual nature of the oxidation-reduction behavior of blue

copper proteins. The goal is to prepare a gene for plastocyanin that can later be easily manipulated to produce structural variants of the protein. These variants should aid in the study of the molecular basis of the functional properties of plastocyanin.

The system described in this thesis for the design and synthesis of a gene for poplar leaf plastocyanin, has been subsequently followed for the construction of two other synthetic genes in our laboratory.

Background of Plastocyanin

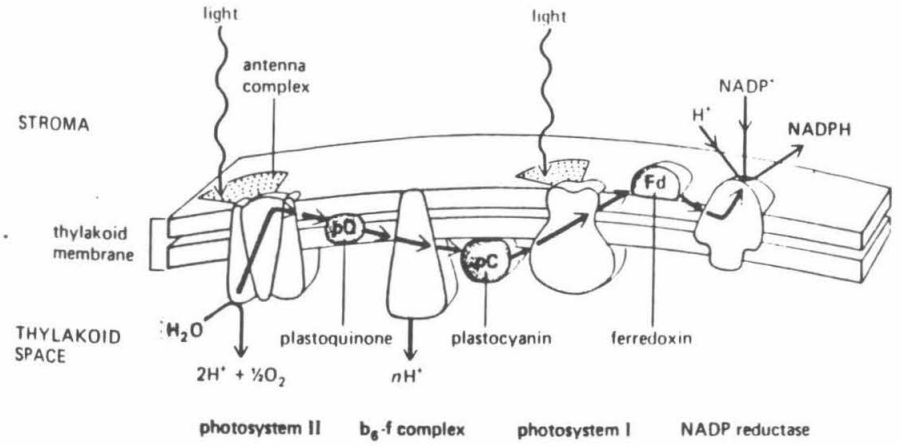
Function of Blue Copper Proteins

Azurin and plastocyanin are blue copper proteins (1, 2) with a single polypeptide chain of 11,000 to 14,000 daltons. Blue copper proteins are found in some bacteria where they participate in electron transfer chains prior to the final oxidation step. They also occur in plants and algae where they are essential to photosynthesis. The azurins are one of the best characterized group of blue copper proteins second only to plastocyanin. These proteins are found in some bacteria where they have an electron transfer function, although it is not clear exactly what that function is (3).

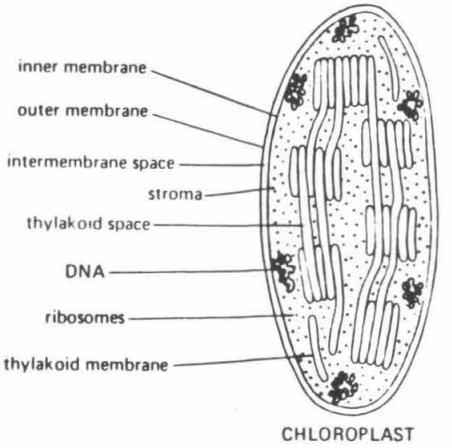
Plastocyanin is functional in the lumen of chloroplasts, as shown in Figure 1, where it is the final electron carrier from photosystem II to I in photosynthesis (4-6). The electron is transferred from cytochrome C_{552} (f) (+360 mV) → plastocyanin (+370 mV) → oxidized P700 (+400-500 mV), the final reaction center of photosystem I. The characteristic feature of these blue copper proteins is possession of one copper atom per molecule which is tightly bound through contacts with side chains of amino acids in the protein chain. This copper atom is the site for the electron transfer activity of the proteins. In the oxidized state, these proteins show an intense blue color. Plastocyanin from poplar leaves (99 amino acids) and azurin from two bacterial sources *Pseudomonas aeruginosa* and *Alcaligenes dentrificans* (129 amino acids) have been crystallized and their three-dimensional structures established (9-16). Poplar leaf plastocyanin is shown in Figure 2 (17).

Properties of Blue Copper Proteins

The most obvious spectral feature of the blue copper proteins is the very intense absorption band peaking at about 600 nm with a molar absorption coefficient, $\varepsilon \approx 4500\ M^{-1}cm^{-1}$. This absorption gives blue copper proteins



(a) Diagram illustrating the orientation of the principal membrane components involved in photosynthesis. Electrons pass from cytochrome f to plastocyanin (PC) and are transferred to photosystem I.



(b) Figure showing the location of the thylakoid membrane within the the chloroplast.

Figure 1

Cu(II) Poplar leaf plastocyanin. The figure was drawn on the Evans and Sutherland graphics system using coordinates from the Brookhaven Protein Bank, Brookhaven National Laboratory.

Figure 2

their intense coloration and has been assigned to a cysteine sulfur to copper charge-transfer (18-20). Another unique spectroscopic property is the unusually narrow hyperfine splitting from the copper nucleus in the epr spectrum. The reasons for this reduced hyperfine splitting are still being debated (21, 22). Another important property of the blue copper site is the relatively positive reduction potential. This reduction potential is believed to be the result of a strained geometry around the copper atom (23, 24). This strain around the copper site is thought to be a result of the protein conformation (20, 25, 26) and is thought to produce facile outer-sphere electron transfer kinetics. Another possibility is that the structure of the blue copper site is used to discriminate against other metals and promote the specific uptake of copper. Since zinc and iron are more abundant in the biological environment they would be expected to compete for the metal binding site in blue copper proteins (23). Because of the structural and functional information available (5, 6) and the important energy related processes in which they are involved (27, 28), plastocyanin and azurin provide an excellent opportunity for studies of structure/function relationships of blue copper proteins. Some of the properties of plastocyanin, azurin and other blue copper proteins are outlined in Table 1.

Structure of Plastocyanin and Azurin

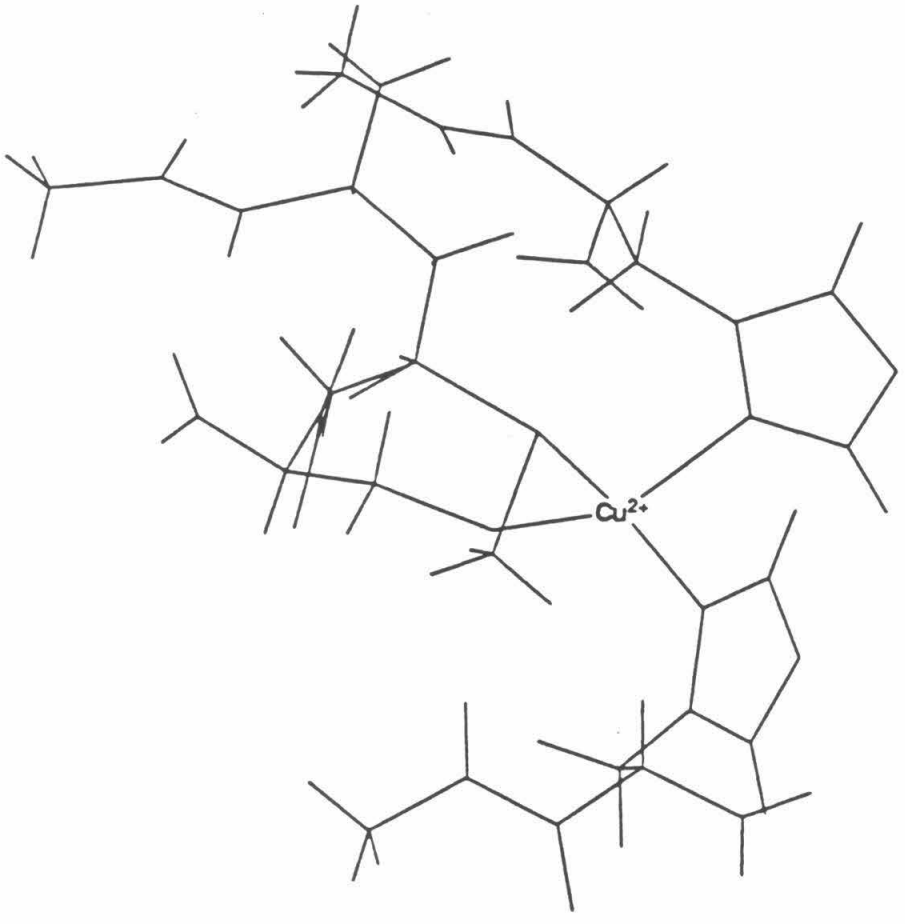
In poplar leaf plastocyanin the ligands to the copper are the thiolate sulfer of Cys 84 (Cu-S, 2.13 Å), the imidazole nitrogens of His 37 (Cu-N, 2.04 Å) and His 87 (Cu-N, 2.10 Å) and the thioether sulfer of Met 92 (Cu-S, 2.90 Å) (3-5). The copper binding site of plastocyanin is shown in Figure 3 (17). This copper site has been reported as a distorted tetrahedral arrangement, however the thioether (Met 92) distance is long enough that one might question its relevance (29).

A Comparison of Properties of Single Blue Copper Proteins

Protein	Source	M.W.	pI	E°/mV (pH)	λmax (ε)/nm ($M^{-1}cm^{-1}$)
Plastocyanin	Chloroplasts, plants/algae	10,500	4.2	370 (7.0)	597 (4,500)
Azurin	Bacteria	14,000	5.4	330 (6.4) 350 (5.0)	625 (4,800)
Stellacyanin	Lacquer tree	20,000	9.86	184 (7.1)	608 (4,080)
Rusticyanin	Thiobac ferro oxidans	16,000	9.1	680 (2.0)	597 (1,950)
Umecyanin	Horseradish roots		5.85	283 (7.0)	610 (3,400)

(table rewritten from reference 22)

Table I



Copper binding site from poplar leaf plastocyanin. The figure was drawn on the Evans and Sutherland graphics system using coordinates from the Protein Data Bank, Brookhaven National Laboratory.

Figure 3

In the azurin from *Alcaligenes dentrificans* crystal structure, which has recently been refined to 1.8 Å, the copper atom makes three strong bonds with the thiolate sulfur of Cys 112 (Cu-S, 2.13 Å) and the imidazole nitrogens of His 46 (Cu-N, 2.06 Å) and His 117 (Cu-N, 1.96 Å) (9). These three ligands form a distorted trigonal-planar arrangement about the copper atom. Much longer contacts to the copper atom are made with the thioether-sulfer of Met 121 (Cu-S, 3.13 Å) and the peptide carbonyl oxygen of Gly 45 (Cu-O, 3.11 Å). These contacts would distort the site to an axially elongated trigonal-bipyramidal arrangement. Therefore, the coordination geometry may be described as trigonal planar, trigonal pyramidal, or trigonal bipyramidal depending on whether the Met (Cu-S) and Gly (Cu-O) approaches are considered as weak bonds.

It may be crucial to distinguish between these arrangements of the copper site in order to understand the unique spectroscopic and functional properties of plastocyanin, azurin and other blue copper proteins. Because of its smaller size, and also because of its involvement in photosynthesis, our attention for the first construction of a synthetic gene in our laboratory was focused on poplar leaf plastocyanin.

Background of Gene Synthesis

General Techniques

Over a period of some 20 years, a series of interactions between genetics, biochemistry and microbiology has led to the development of a new technology which has made possible the transfer of a gene (30, 31) or a small cluster of genes, on a segment of DNA from almost any organism to one of the standard and easily grown laboratory organisms; the most conspicuous organism being the bacterium *Escherichia coli*. A host of supplementary techniques permit the regulation of the expression of the transferred genes (32-35), so that the proteins they specify may be synthesized very efficiently in the bacterium. This allows the protein to be produced cheaply and abundantly. In order to produce desired proteins using this recombinant DNA technology, one must either isolate or synthesize the gene that encodes that particular protein. The advantage of total synthesis is the opportunity to engineer desired features into the DNA such as: restriction sites, regulatory signals for transcription and/or translation and usage of the most abundant tRNA codons for a given organism (36).

Examples of Gene Synthesis

The first gene synthesis was carried out by Khorana and coworkers in the 1960s with the yeast alanine tRNA gene (37). The key concept in Khorana's work is the inherent ability of DNA to base pair (38). Also a major factor to his success was the discovery of DNA joining enzymes and polynucleotide kinase (39-41). Khorana formulated and followed a three step approach (42): (1) chemical synthesis of short oligonucleotides, (2) enzymatic phosphorylation of 5′ OH end-groups to encourage joining, and (3) ligase-catalyzed joining of hydrogen bonded duplexes.

Several genes have since been synthesized using this approach some examples of which are the following: In 1977, Riggs, Itakura and coworkers synthesized the gene for somatostatin (43) and fused this to the gene for β-galactosidase in the plasmid pBR322 (44-46). This represents the first recovery of a functional polypeptide product from chemically synthesized DNA. The synthesis required for somatostatin consisted of eight oligonucleotides with five base complementary overlaps for efficient oligonucleotidejoining.

Two of the longest genes to be synthesized include human leukocyte interferon α-1 (47) which is 514 base pairs (166 amino acids) and bovine rhodopsin (48) which is 1057 base pairs (348 amino acids). These are sections of DNA which include initiation and termination codons and restriction enzyme sites for insertion into a plasmid. The synthesis for human leukocyte interferon α-1 requires 67 oligonucleotides with an average length of 15 nucleotide residues. The synthesis for bovine rhodopsin required 72 synthetic oligonucleotides with average lengths of 15-40 nucleotide residues. An alternate approach was developed by Itakura, Rossi and coworkers for synthesis of a 132 base pair segment coding for amino acids 126-stop of human leukocyte interferon α-2 (49). This method involves synthesis of oligonucleotides which are annealed to form partial duplex structures. These duplexes are then used as a substrate for DNA polymerase I (Klenow) (50) and the four 2′-deoxynucleotide 5′-triphosphates. These segments are digested with the appropriate restriction endonucleases for insertion into the plasmid and the final step is a blunt end ligation to close the plasmid. This approach reduces the number of synthetic oligonucleotides required, however there is an increase in enzymatic manipulations prior to ligation into the plasmid.

With the introduction With the inof automated DNA synthesis the number of oligonucleotides necessary for gene construction is no longer a major concern.

Cassette Mutagenesis

Two of the most recent examples of synthetic genes also contain unique restriction sites placed at intervals throughout the gene in order to facilitate cassette mutagenesis (51-53).

Cassette mutagenesis allows several mutations to be introduced at a target site in a single experiment. When the necessary restriction sites are available, cassette mutagenesis offers an efficient alternative to oligonucleotide-directed mutagenesis (54, 55). These synthetic genes code for the human complement fragment C_{5a} (56) and calmodulin (57).

Design of System

General Design

The synthesis of a gene to code for the blue copper protein plastocyanin employed variations of previously reported methods along with some new techniques developed during this project. The strategy for the construction of the gene for plastocyanin gene is outlined below.

(1) DNA sequence. Since the DNA sequence is not known for poplar leaf plastocyanin, it was generated from the known 99 amino acid sequence (58).

(2) Restriction site search of the possible DNA sequences. Unique restriction enzyme recognition sites are necessary for construction of the gene and for the introduction of mutations into the completed gene using cassette mutagenesis.

(3) Homology search on the oligonucleotides used for construction of the gene. This avoids self-complementary regions or complementary regions with other oligonucleotides other than those intended for the formation of duplex structures.

(4) Four step construction of the gene. The gene is constructed in four steps from both ends towards the middle. The introduction of the unique restriction sites facilitates this method of gene construction.

The synthetic gene uses those codons preferred by *E. coli* (36) whenever possible, although some changes are required in order to introduce restriction sites into the gene and/or reduce homologous sequences. The vector used for the gene construction was pBR322 (45) in which the gene was introduced between the Eco RI and Ava I restriction enzyme sites. The strategy for producing the synthetic gene involves construction of the gene from both ends toward the middle. Using unique restriction sites engineered into the

structural gene for poplar leaf plastocyanin newly synthesized elements of the gene may be inserted.

Polymerization Strategy for Plastocyanin Gene Construction

Initially the strategy involved the chemical synthesis of two pairs of oligonucleotides with a short region of overlap followed by *in vitro* enzymatic manipulations to complete the duplex structures (59). These oligonucleotides are designed to contain a ten base pair overlap region to form a partial duplex structure allowing the rest of the duplex to be filled in using DNA polymerase 1 (Klenow) and the four 2′-deoxynucleotide 5′-triphosphates (dNTPs). Restriction site overhangs can then be created using the exonuclease activity of T4 polymerase and the appropriate dNTP(s) to allow digestion of the DNA from the 3′ end to a specific base leaving the desired overhang for subsequent ligation reactions as shown in Figure 4. This strategy was developed to limit the number of oligonucleotides which needed to be chemically synthesized as automated DNA synthesis was not routinely available at the time this project was begun. This polymerization followed by exonuclease strategy met with limited success which led to a new strategy for the plastocyanin gene construction.

Plastocyanin Gene Construction: Total Synthesis

A new experimental design was developed to take advantage of the rapid improvements and availability of automated DNA synthesis which allows efficient synthesis of oligonucleotides in high yields. This new scheme for the synthesis of a gene for plastocyanin involves the synthesis of oligonucleotides for both strands of the entire gene. These oligonucleotides are introduced in a stepwise manner (*vide infra*). No polymerization or exonuclease reactions are required thus simplifying the procedure. The oligonucleotides are synthesized in sets of four, two pairs of complementary oligonucleotides which are

Figure 4

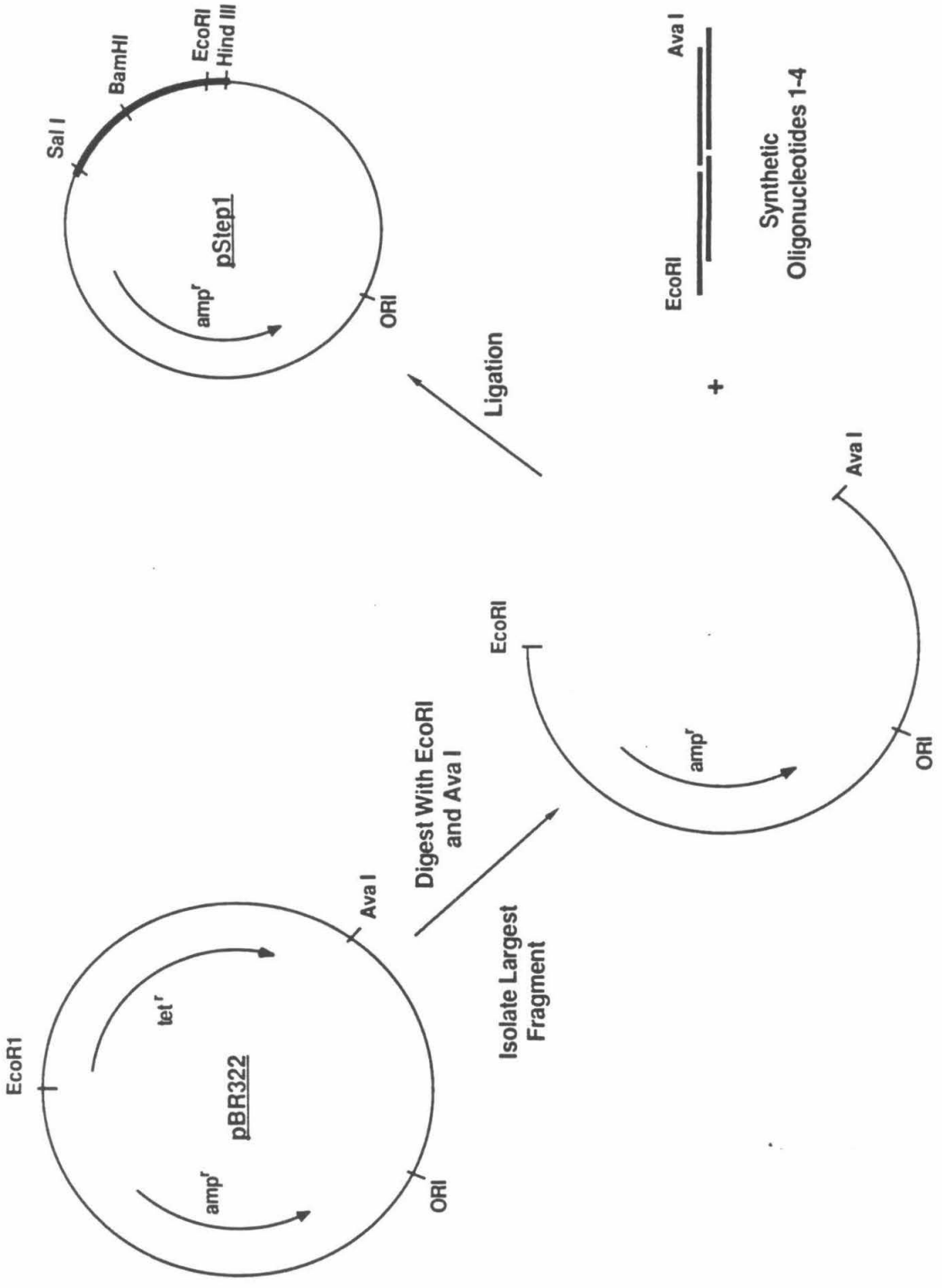
Oligonucleotides 1P and 2P were synthesized manually, 3P-8P were synthesized on an ABI automated DNA synthesizer. The oligonucleotides were annealed to their complementary partner and Klenow enzyme was used for completion of the second strand as shown. Oligonucleotides 1P-4P were to be used in the first synthesis step following an exonuclease reaction to create the desired overhang. For the 1P-2P duplex T4-polymerase was used in a reaction with dCTP to remove only the boxed bases from the 3′ end. For the 3P-4P duplex T4-DNA polymerase was used in a reaction with T4-DNA polymerase and dATP to remove only the boxed bases from the 3′ end. These duplexes were then to be cloned into pBR322 between Eco RI and Ava I. Oligonucleotides 5P-8P were designed for the second step of the gene synthesis. Following annealing and polymerization, the oligonucleotides were to be digested with Eco RI and Hind III to create the correct overhangs for cloning the duplexes between the Eco RI and Hind III sites of the plasmid recovered from step 1.

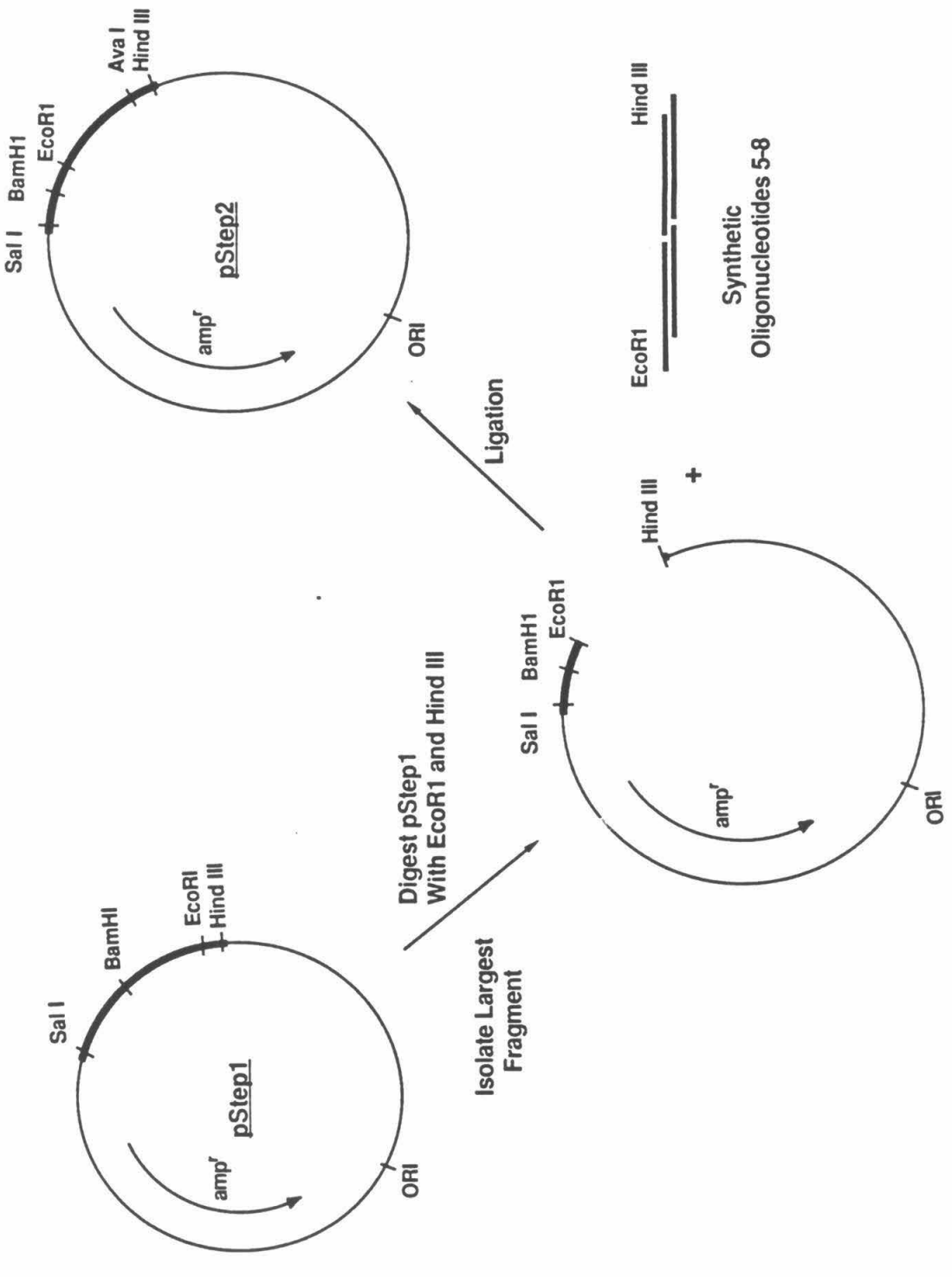

Eco RI overhang
5'- A A T T G G T C G A C A T G A T C G A C G T T C T G C T G G G T G C A G A C G A C G G A T C C C -3'
3'- T T A A C C A G C T G T A C T A G C T G C A A G A C G A C C C A C G T C T G C T G C C T A G G G -5'
1 P
2 P
Eco RI
Hind III
Ava I overhang
5'- T G G C A T T C G T T C C G T C C G A A T T C A A A A G C T T A T C G G -3'
3'- A C C G T A A G C A A G G C A G G C T T A A G T T T T C G A A T A G C C -5'
3 P
4 P
Eco RI
5'- A G G A A T T C T C C A T C T C T C C G G G T G A A A A A A T C G T A T T C A A A A A C A A C G C C -3'
3'- T C C T T A A G A G G T A G A G A G G C C C A C T T T T T T A G C A T A A G T T T T T G T T G C G G -5'
5 P
6 P
Hind III
5'- G G T T T C C C G C A C A A C A T C G T T T T T G A C G A A G A C T C C A T C C C G A G A A A A G C T T G G -3'
3'- C C A A A G G G C G T G T T G T A G C A A A A A C T G C T T C T G A G G T A G G G C T C T T T T C G A A C C -5'
7 P
8 P

annealed together to produce two double stranded fragments. These duplex fragments contain single stranded overhang regions on each end to allow ligation of the fragments to each other and simultaneously into the cloning vector, as shown in Figures 5 and 6. The products of these *in vitro* steps are then used to transform *E. coli* for the purpose of amplifying the amount of plasmid recovered from each step (60, 61). About 70-90 base pairs are introduced at each step. In the subsequent step, restriction sites specifically incorporated into the DNA sequence are opened to serve as places for insertion of new DNA. The gene for plastocyanin, 297 base pairs was synthesized in four such steps. Cloning the gene for plastocyanin between the Eco RI and Ava I restriction sites in pBR322 leaves the gene for β-lactamase intact thereby allowing selection of *E. coli*, on the basis of resistance to ampicillin. The plasmid DNA recovered after each step from these ampicillin resistant colonies of *E. coli* can be analyzed for the presence of gene inserts using restriction enzymes. At least two unique restriction enzyme sites are introduced into the plasmid at each step (except the final step); these sites are necessary to clone the next fragments of DNA into the vector. Ten base pairs of non-coding DNA were placed between the two unique restriction sites used to open the plasmid for the next cloning step to allow for maximum cleavage by the enzymes. For a typical 6 base recognition sequence, all 6 bases are required for the enzyme to cleave the DNA, however at most only 5 of these bases are left in the large fragment of plasmid DNA following cleavage. This allows one to choose the nucleotide to be placed in the sixth position of the recognition sequence with the insertion of the new DNA. Therefore, the recognition sequence may be retained or destroyed at that position in the gene sequence. This permits one to use the same restriction enzyme more than once in the gene construction while still maintaining it as a unique restriction

Figure 5

Schematic representation of the strategy used for the synthesis of a gene for poplar leaf plastocyanin. At each step two oligonucleotide duplexes were ligated into the plasmid which had been digested with the appropriate restriction enzymes. Two unique restriction sites are designed into each new DNA insert to allow the plasmid to be opened and another set of oligonucleotide duplexes inserted. This stepwise construction allows the plasmid to be characterized at each step and should facilitate the synthesis of very long genes.


Sal I
BamHI
EcoRI
Hind III
pStep1
amp^r
ORI
Ligation
EcoRI
Ava I
Synthetic
Oligonucleotides 1-4
+
EcoR1
tet^r
pBR322
amp^r
ORI
Ava I
Digest With EcoRI
and Ava I
Isolate Largest
Fragment
EcoRI
amp^r
ORI
Ava I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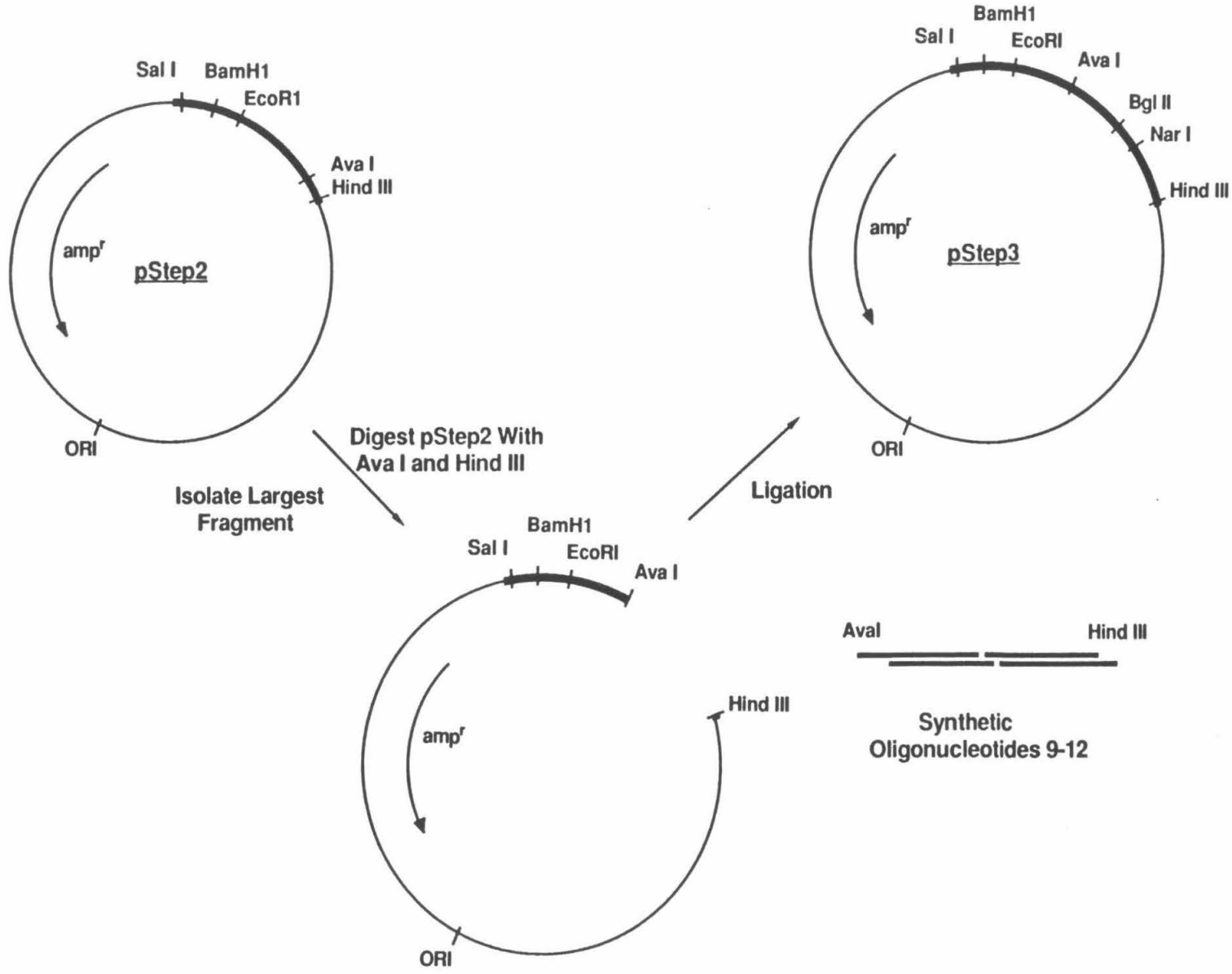
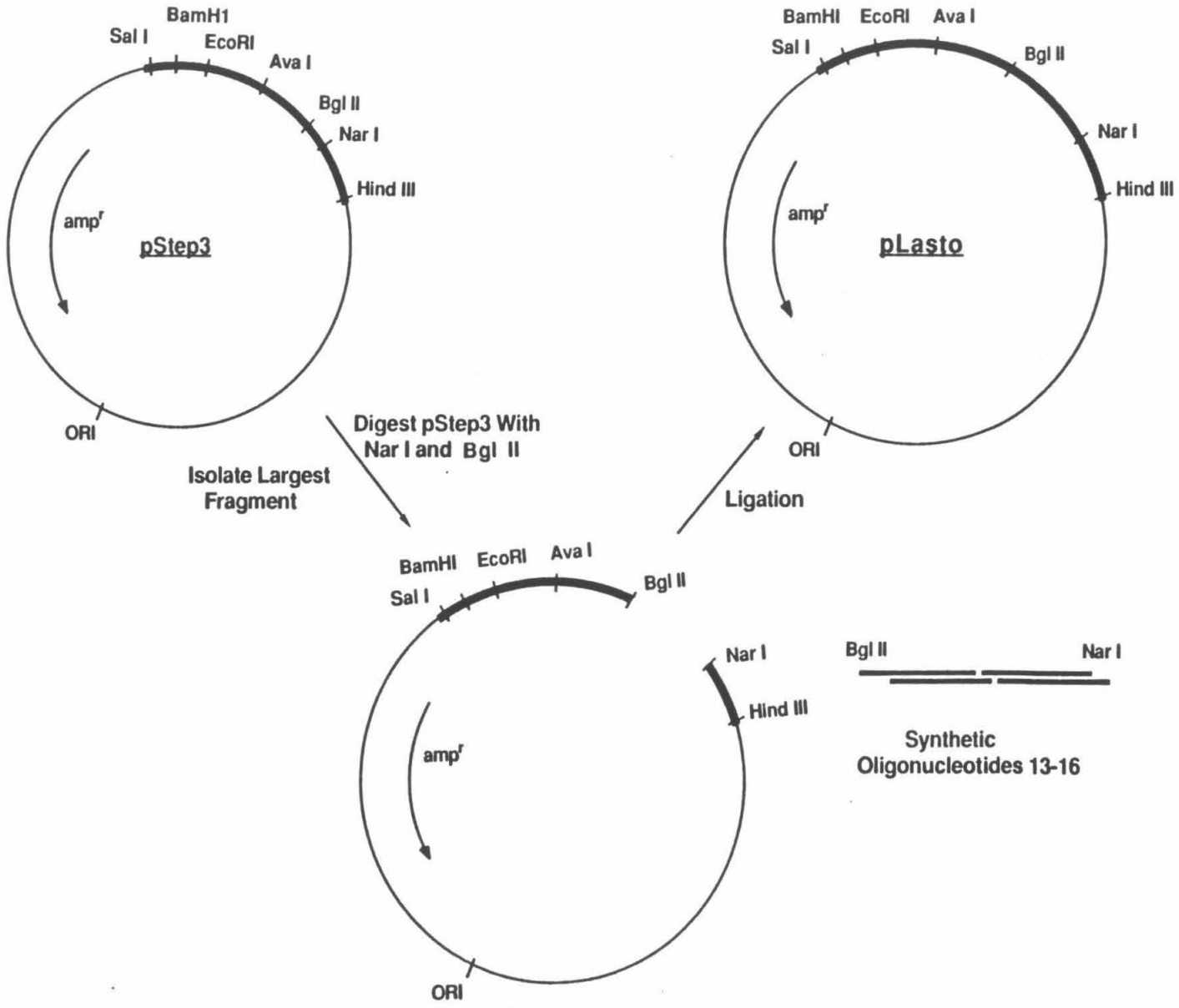
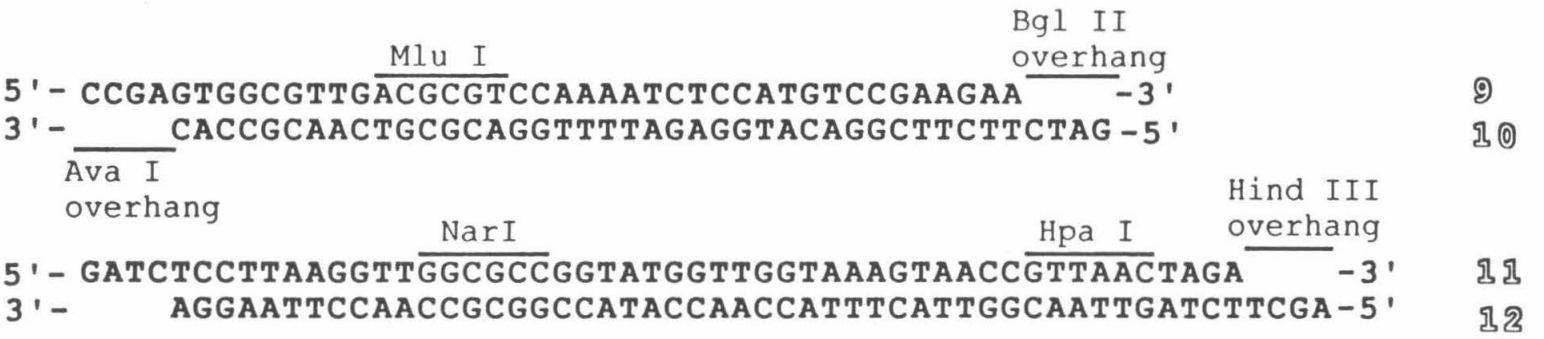
BamHI
EcoRI
Hind III
pStep1
amp^r
ORI
Digest pStep1
With EcoR1 and Hind III
Isolate Largest
Fragment
Sal I
BamH1
EcoR1
Hind III
amp^r
ORI
+
Hind III
EcoR1
Synthetic
Oligonucleotides 5-8
Ligation
Sal I
BamH1
EcoR1
Ava I
Hind III
pStep2
amp^r
ORI

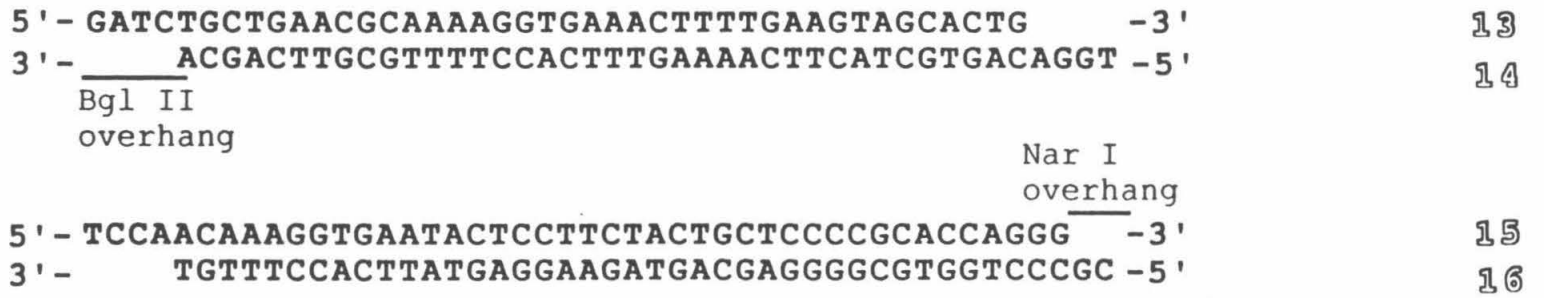

Sal I
BamH1
EcoR1
Ava I
Hind III
amp^r
pStep2
ORI
Digest pStep2 With
Ava I and Hind III
Isolate Largest
Fragment
BamH1
Sal I
EcoRI
Ava I
Hind III
amp^r
ORI
Ligation
BamH1
Sal I
EcoRI
Ava I
Bgl II
Nar I
Hind III
amp^r
pStep3
ORI
AvaI
Hind III
Synthetic
Oligonucleotides 9-12


BamH1
Sal I
EcoRI
Ava I
Bgl II
Nar I
Hind III
amp^r
pStep3
ORI
Digest pStep3 With
Nar I and Bgl II
Isolate Largest
Fragment
BamHI EcoRI Ava I
Sal I
Bgl II
Nar I
Hind III
pLasto
ORI
Ligation
BamHI EcoRI Ava I
Sal I
Bgl II
Nar I
Hind III
amp^r
ORI
Bgl II
Nar I
Synthetic
Oligonucleotides 13-16

The DNA sequence of oligonucleotides 1-16 used in the synthesis of a gene for poplar leaf plastocyanin

Figure 6


Mlu I
Bgl II
overhang
5'- CCGAGTGGCGTTGACGCGTCCAAAATCTCCATGTCCGAAGAA -3' 9
3'- CACCGCAACTGCGCAGGTTTTAGAGGTACAGGCTTCTTCTAG -5' 10
Ava I
overhang
NarI
Hpa I
Hind III
overhang
5'- GATCTCCTTAAGGTTGGCGCCGGTATGGTTGGTAAAGTAACCGTTAACTAGA -3' 11
3'- AGGAATTCCAACCGCGGCCATACCAACCATTTCATTGGCAATTGATCTTCGA -5' 12


5'- GATCTGCTGAACGCAAAAGGTGAAACTTTTGAAGTAGCACTG -3' 13
3'- ACGACTTGCGTTTTCCACTTTGAAAACTTCATCGTGACAGGT -5' 14
Bgl II
overhang
Nar I
overhang
5'- TCCAACAAAGGTGAATACTCCTTCTACTGCTCCCCGCACCAGGG -3' 15
3'- TGTTTCCACTTATGAGGAAGATGACGAGGGGCGTGGTCCCGC -5' 16

site in the final gene sequence (only where last used for construction). The recycling of certain restriction sites and the stepwise manner of gene synthesis allows the construction of very long genes. Plasmid DNA recovered from the transformed cells is screened for these restriction sites on an analytical scale so that a larger scale plasmid preparation, necessary for the next cloning step, can be done on a single colony known to contain the desired insert based on restriction digest analysis.

The stepwise construction is designed to decrease the incidence of mistakes being introduced into the gene by allowing the products to be amplified and screened at each step. This strategy should avoid any extra fragments being ligated into the gene. The completed gene is sequenced completely at which time any mistakes such as base additions, substitutions, or deletions can be corrected using cassette mutagenesis.

The strategy developed during the plastocyanin gene synthesis project and described in this thesis, has subsequently been used in the synthesis of two other genes in these laboratories (62, 63).

Results and Discussion

Oligonucleotide Synthesis and Purification

Manual Synthesis of Oligonucleotides

Oligonucleotides 1 and 2 were synthesized manually using the solid phase phosphoramidite approach as described in the literature (64, 65). An outline of the cycle schedule is given below.

(1) Toluene wash

(2) Dichloroacetic acid cleavage

(3) Diisopropylethylamine

(4) CH_3CN wash

(5) Coupling

(6) CH_3CN wash

(7) Capping reagent

(8) CH_3CH wash

(9) Oxidation

(10) Methanol wash

The overall yield for oligonucleotide 1, a 29 mer, was 24%, as determined by dimethoxytrityl (DMT) cation absorbance at 498 nm; this is approximately 95% at each cycle. Overall yield for oligonucleotide 2, also a 29 mer, was 39.4% or approximately 96.8% for each cycle.

The oligonucleotides were deprotected according to the schedule shown below.

(1) Thiophenol:triethylamine:dioxane (1:2:2)

(2) Wash dioxane, methanol, ether

(3) Dry

(4) Saturated ammonium hydroxide (50°C overnight)

(5) Evaporation under vacuum

(6) Acetic acid

(7) Evaporation under vacuum

The procedure for DNA synthesis is outlined in Figures 7-9.

Automated Oligonucleotide Synthesis

All subsequent oligonucleotides were synthesized using an ABI automated DNA synthesizer. The chemistry for the automated synthesis is analogous to the phosphoramidate chemistry described above with minor modifications (66). The automatic DNA synthesizer also employs an automatic deprotection cycle.

HPLC Purification of Oligonucleotides

Oligonucleotides 1P, 2P and 3P were purified on a C_{18} reverse phase HPLC column. Conditions were optimized for separation in each case using a linear gradient of acetonitrile in 0.1 M TEAAc.

Oligonucleotide 1P 12-13% over 1 hour

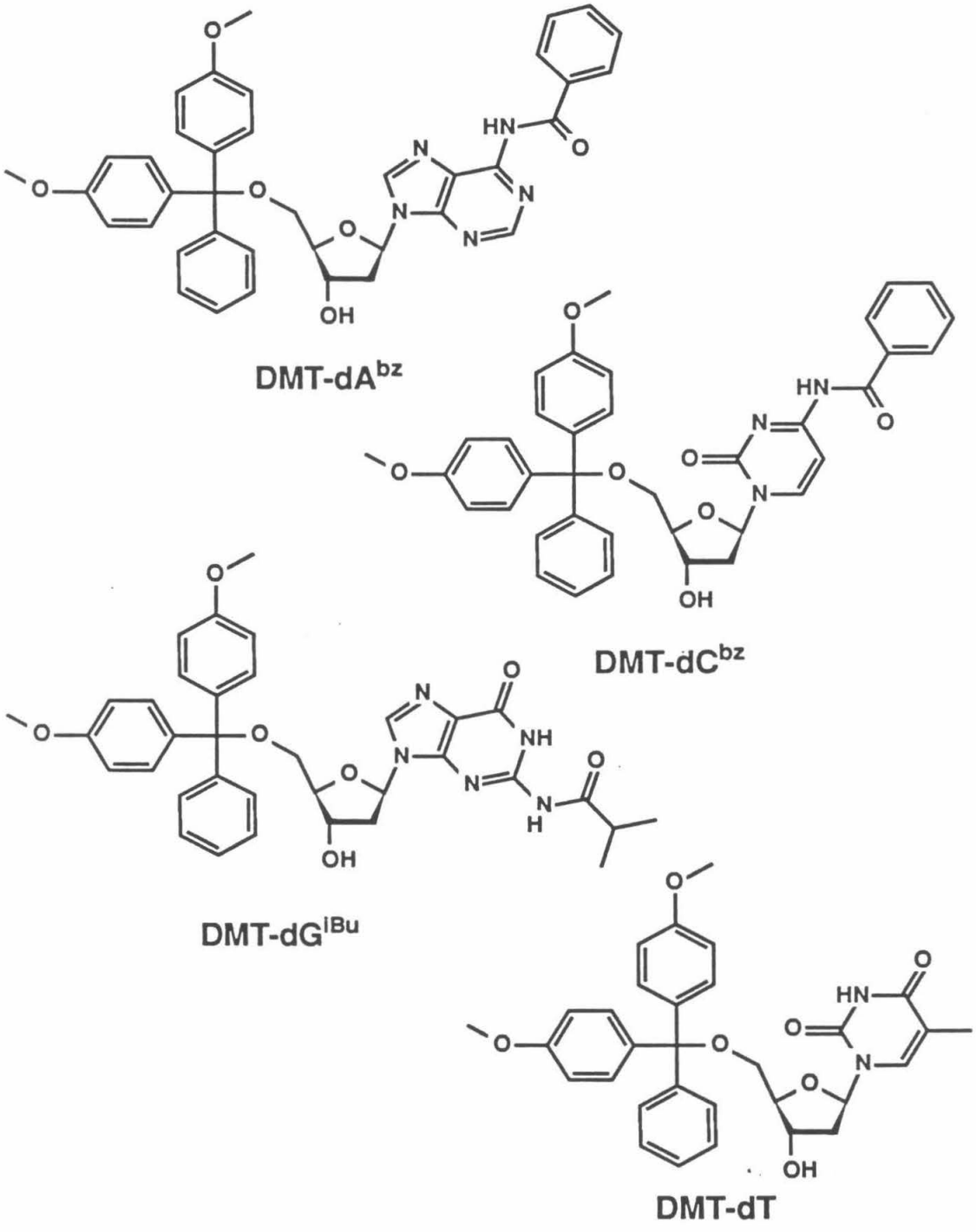
Oligonucleotide 2P 12-13% over 1 hour

Oligonucleotide 3P 11-13% over 1 hours, 15 minutes

Approximately 0.05-0.07 μmoles of each sample was loaded onto the column and the appropriate peak collected. The solvent was then evaporated under vacuum, resuspended in H_2O and lyophilized to dryness.

Gel Purification of Oligonucleotides

Oligonucleotide 3P as well as all subsequent oligonucleotides were purified using a 15% 7 M urea denaturing polyacrylamide gel. The DNA was visualized using a fluorescent thin layer chromatography plate beneath the gel and illuminating with short wave UV light. The desired band is excised from the gel, eluted from the polyacrylamide in 0.2 M NaCl and purified further using G-25-150 Sephadex in order to exchange the buffer.



Protected bases used in the synthesis of oligonucleotides

Figure 7

Condensation Cycle

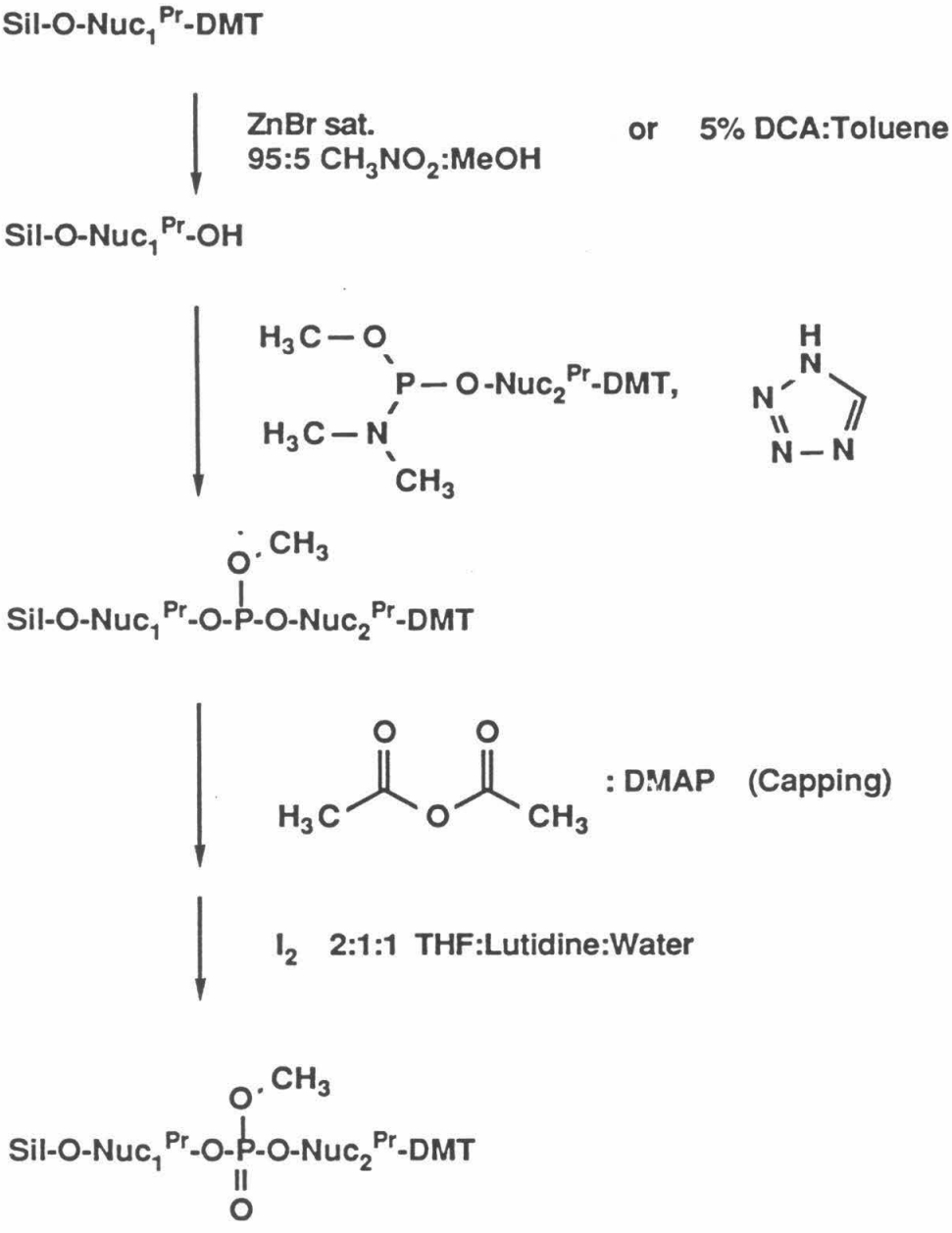


Figure 8

Deprotection Cycle

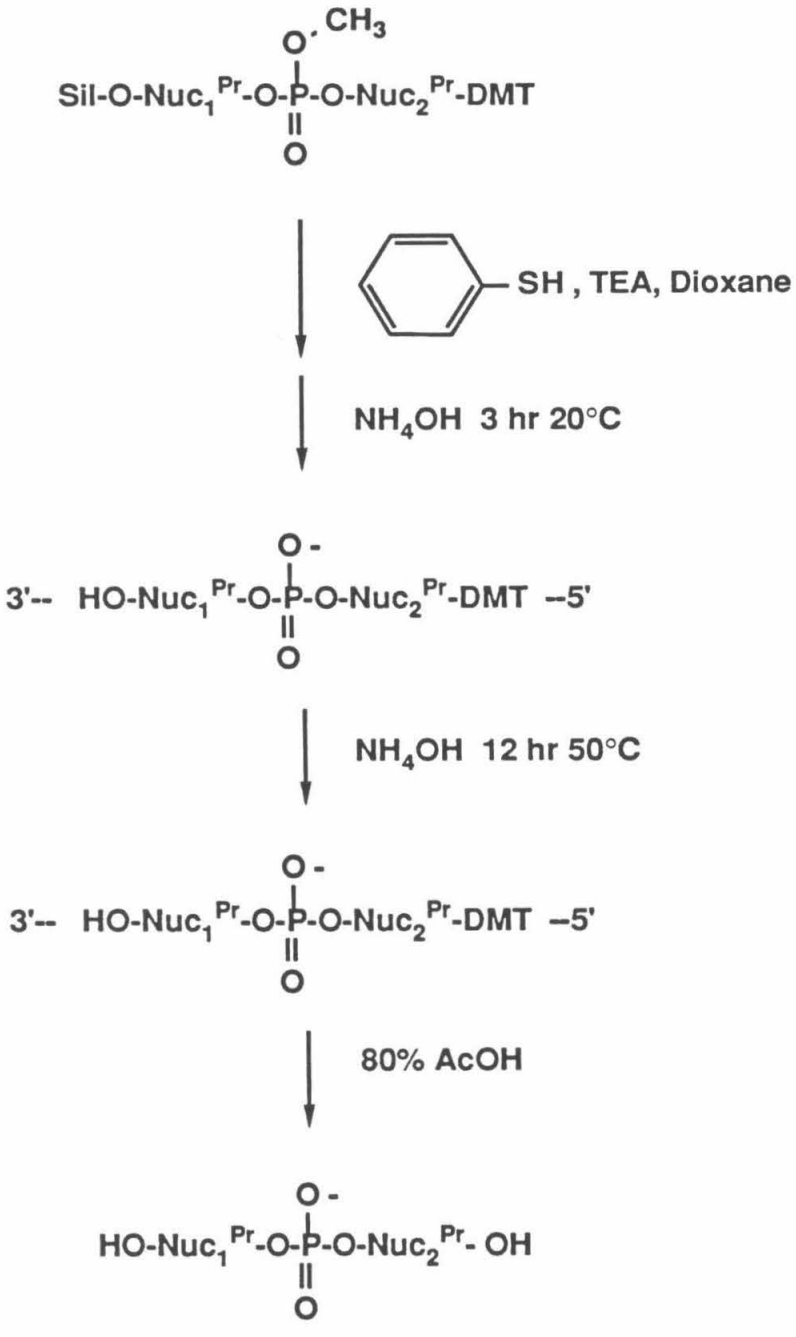


Figure 9

Polymerization Strategy for Plastocyanin Gene Construction

5' End Labelling of Oligonucleotides

Unpurified oligonucleotides 1P, 2P, 3P and 4P and purified oligonucleotides 1P, 2P, 3P and 4P were individually phosphorylated at their 5' termini with γ^{32}P-adenosine 5'-triphosphate (γ^{32}P-ATP). Equimolar amounts of purified phosphorylated oligonucleotides were annealed to their complementary unphosphorylated partners.

Annealing of Oligonucleotides

The oligonucleotides were annealed together by heating to 90°C for 3 minutes and allowing them to cool slowly to room temperature in the water bath.

Polymerization Reactions for Second Strand Synthesis

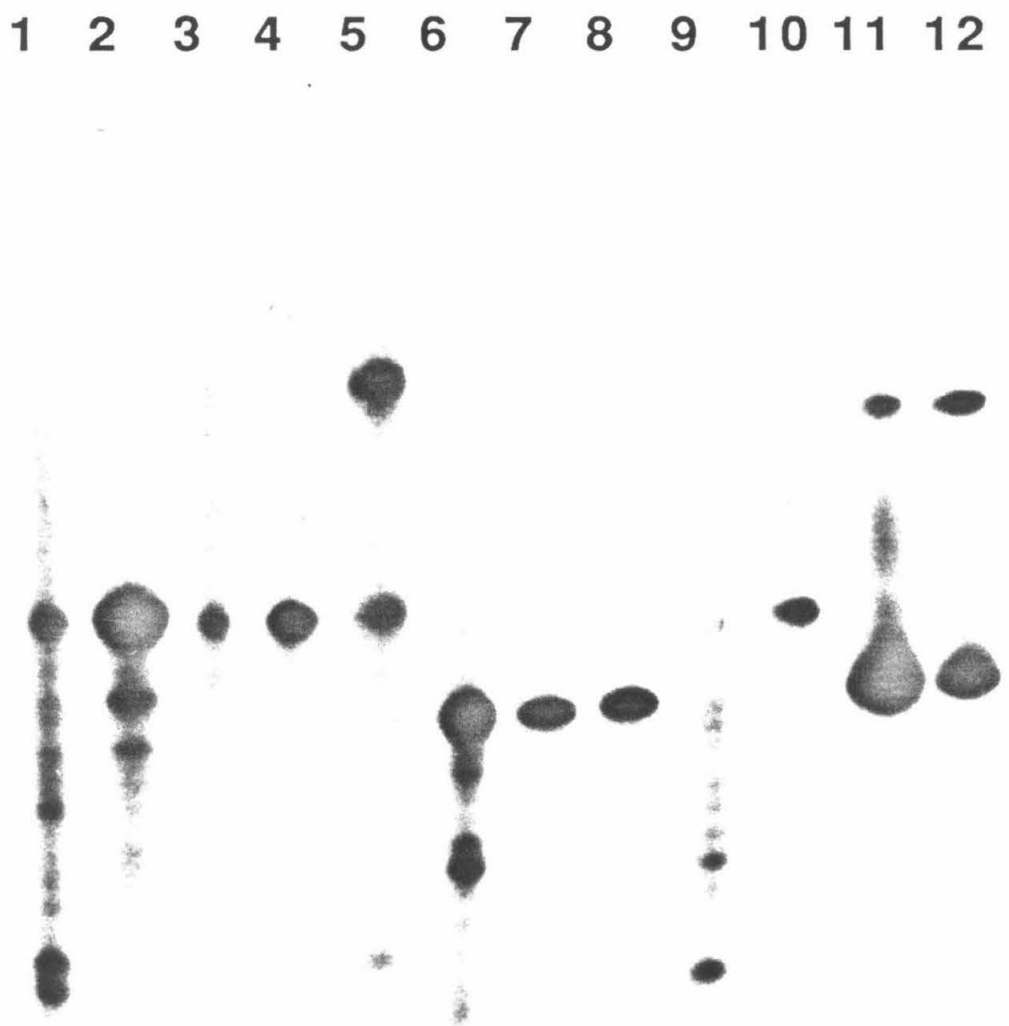
The polymerization reactions were performed on the partial duplex structures with the four 2'-deoxyribonucleotide 5'-triphosphates as substrate. Aliquots of the individual oligonucleotides and the polymerized products were electrophoresed on a 15% polyacrylamide 7 M urea denaturing gel as shown in Figure 10. The polymerization reactions appeared to proceed sufficiently in order to carry on to the exonuclease reactions on an analytical scale.

Exonuclease Reactions

Following the polymerase reactions an ethanol precipitation was performed. The pellets were dissolved in the appropriate buffer and exonuclease reactions were initiated with the large fragment of DNA polymerase I (Klenow). Only one of the four 2'-deoxynucleotide 5'-triphosphates was added to the reactions in order to result in specific overhang as outlined in Figure 4. The reactions were analyzed on a 20% polyacrylamide 7 M urea denaturing gel to separate the oligonucleotides and look for a change in the length of the two oligonucleotides. After several

Figure 10

Klenow enzyme fill-in reactions of oligonucleotides 1P-4P. Autoradiogram of a 15% denaturing polyacrylamide gel. Oligonucleotides were radioactively labelled at the 5' end with T4-polynucleotide kinase and γ^{32}P-ATP. Oligonucleotides were annealed by heating to 90°C for 3 minutes and cooling slowly. Polymerization reactions were carried out at room temperature for 30 minutes. **Lane 1.** Oligonucleotide 1P prior to purification. **Lane 2.** Oligonucleotide 1P after purification by HPLC. **Lane 3.** Oligonucleotide 2P prior to purification. **Lane 4.** Oligonucleotide 2P after purification by HPLC. **Lane 5.** Oligonucleotides 1P and 2P, annealed and filled in, only oligonucleotide 2P was radioactively labelled. **Lane 6.** Oligonucleotide 3P prior to purification. **Lane 7.** Oligonucleotide 3P after purification by HPLC. **Lane 8.** Oligonucleotide 3P after purification by gel electrophoresis. **Lane 9.** Oligonucleotide 4P prior to purification. **Lane 10.** Oligonucleotide 4P after purification by gel electrophoresis. **Lane 11.** Oligonucleotides 3P and 4P annealed and filled-in. Both were purified by gel electrophoresis, only oligonucleotide 3P was radioactively labelled. **Lane 12.** Oligonucleotides 3P and 4P annealed and filled in. Oligonucleotide 3P was purified by HPLC and 4P purified by gel electrophoresis, only oligonucleotide 3P was radioactively labelled.


1 2 3 4 5 6 7 8 9 10 11 12

variations in reaction conditions no exonuclease activity was apparent using Klenow. A polymerase with a stronger 3'-5' exonuclease (67) was thought to be necessary so experiments with T4-polymerase I were initiated. Again the polymerased oligonucleotide duplexes were ethanol precipitated and dissolved in the appropriate buffer and several variations in reaction conditions were tried but no exonuclease activity was apparent when analyzed on a denaturing polyacrylamide gel.

Construction of Plastocyanin Gene: Total Synthesis

Introduction of Automated Oligonucleotide Synthesis

Due to the difficulty in the exonuclease reactions and more importantly the fortunate acquisition of an automated DNA synthesizer by the chemistry department, the approach to constructing the gene for plastocyanin was redesigned. The availability of an automated DNA synthesizer allowed oligonucleotides to be synthesized efficiently and quickly so it was no longer necessary to polymerize the second strand of each duplex. The oligonucleotide could be synthesized easily and the oligonucleotides were designed to contain the appropriate overhangs for ligation to avoid exonuclease reactions and blunt end ligations.

5'-End Labelling and Annealing of Oligonucleotides

Oligonucleotides 1-4 were synthesized as shown in Figure 6. These oligonucleotides were purified on a denaturing polyacrylamide gel. The purified oligonucleotides were analyzed for purity and concentration based on their UV absorbances monitored at 260 and 280 nm (46). Equimolar concentrations of each purified oligonucleotide were individually phosphorylated at their 5' terminus and subsequently annealed to their complementary oligonucleotide (1 annealed to 2 and 3 annealed to 4). This

produced two duplex structures which contained the appropriate overhang regions to allow the initial ligation into pBR322.

Vector Preparations and Ligations

The plasmid pBR322 was digested with the restriction enzymes Ava I and Eco RI; this digested plasmid was used in the ligation without further purification. The ligation was carried out with a ten times molar excess of the duplex oligonucleotide inserts to the digested plasmid. The reaction was allowed to proceed overnight at 15°C. The reaction mixture was then transformed (31) into *E. coli* LS1 cells (all strains of *E. coli* are described at the end of Chapter 2). Transformed LS1 cells were plated onto L-agar plates containing ampicillin and separate plates containing tetracycline for selection. As the tetracycline gene was inactivated by the insert this allowed comparison of the number of colonies on the two plates and therefore an estimate of the percentage of colonies on the ampicillin plate which could be expected to contain the inserted oligonucleotides. This percentage was estimated at approximately 70%. Several colonies were picked from the ampicillin plate and grown overnight in 3 mls of L-broth containing ampicillin. The saturated cultures were then centrifuged to recover a cell pellet from which the plasmid DNA was extracted. The plasmid DNA was screened on the basis of restriction site analysis (*vide infra*). Plasmid pStep 1 was digested with Eco RI and Hind III and oligonucleotides 5-8 were kinased, annealed, ligated into the digested plasmid, and transformed into *E. coli* LS1. This process was continued for Steps 3 and 4 as outlined in Figure 5. A slight variation in protocol was necessary for Step 4 due to incomplete digestion by Nar I. Plasmid DNA pStep 3 was digested with Nar I and the linear band isolated on an agarose gel. The DNA was ethanol precipitated, redissolved in the appropriate buffer and digested with Bgl II. This plasmid was used in the

ligation reaction without further purification. Step 4 produced only 5 colonies after transformation while Steps 1-3 produced ≥100 colonies under the same conditions.

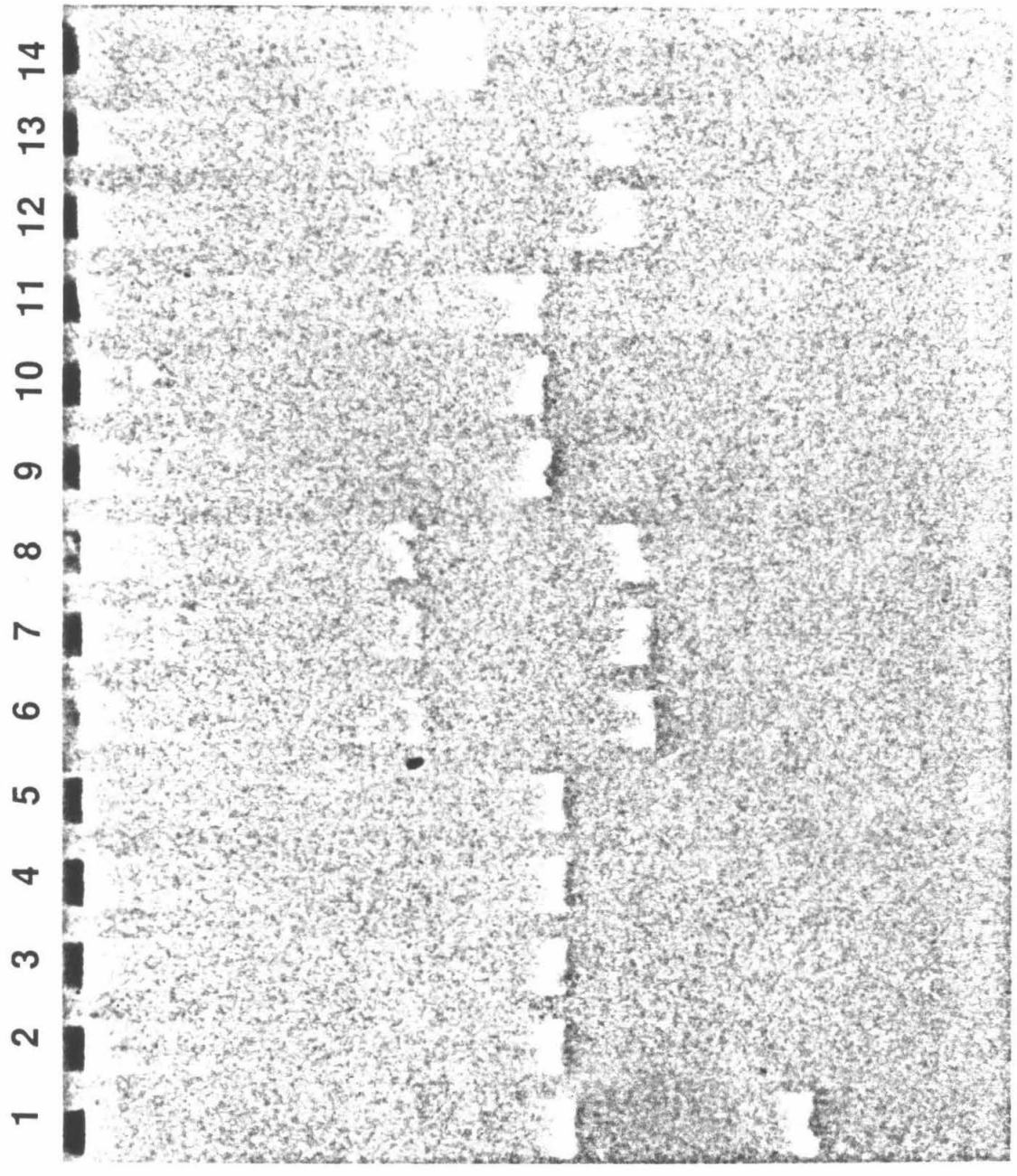
Restriction Site Analyses

With each insertion of a gene fragment (except Step 4) at least two unique restriction sites were introduced into the plasmid; this allowed screening of plasmid DNA following each step. Since the restriction sites are unique to the plasmid, this allowed screening for the gene fragment insert on the basis of a single cutting event. In the first step the original Ava I and Eco RI sites of pBR322 were destroyed when the gene insert was cloned into the plasmid. A new Eco RI site was created within the gene insert as well as Bam HI, Sal I and Hind III sites. The plasmid pStep 1 was screened on the basis of the presence of these restriction sites as shown in Figure 11. This was taken as evidence that the first gene fragment insert had successfully been incorporated into the plasmid. It was decided at this point that DNA sequencing was not required at each step but rather the entire gene would be sequenced upon its completion. At this time any mistakes in the gene sequence could be corrected using cassette mutagenesis for which the system was designed.

In pStep 2 the two oligonucleotide duplexes add an Ava I site and a Nae I site to the plasmid. For pStep 3 the two oligonucleotide duplexes added a Nar I site, Mlu I site, Hpa I site, and a Bgl II site. In pStep 4 no new restriction sites were introduced into the plasmid so analysis of plasmid DNA believed to contain the oligonucleotide insert was based on a size difference. Plasmid DNA pStep 3 and pStep 4 were digested with Pst I followed by Hind III for comparison. The doubly digested plasmid would be expected to give fragments of 943 base pairs and 2184 base pairs without the last insert and

Figure 11

Restriction digest analysis for the first step of gene construction. 1.2% agarose gel stained with ethidium bromide. In step 1 Ava I, Bam HI, Eco RI and Hind III restriction sites were added to the plasmid while the Ava I and Eco RI sites were destroyed. **Lane 1.** pBR322 digested with Eco RI and Ava I. **Lane 2.** pStep 1 digested with Eco RI. **Lane 3.** pStep 1 digested with Hind III. **Lane 4.** pStep 1 digested with Sal I. **Lane 5.** pStep 1 digested with Bam HI. **Lane 6.** pStep 1 digested with Ava I. **Lane 7.** pStep 1. **Lane 8.** Colony 16 (another plasmid from step 1) digested with Eco RI. **Lane 9.** Colony 16 digested with Hind III. **Lane 10.** Colony 16 digested with Sal I. **Lane 11.** Colony 16 digested with Bam HI. **Lane 12.** Colony 16 digested with Ava I. **Lane 13.** Colony 16. **Lane 14.** pBR322 digested with Bam HI.


1 2 3 4 5 6 7 8 9 10 11 12 13 14

fragments of 1043 base pairs an 2184 base pairs with the last two oligonucleotide duplexes inserted. About half of the plasmids analyzed contained the last insert based on this size difference. Figures 12 through 15 show some of the agarose gels used to analyze plasmid pStep 2-pStep 4 with restriction enzymes.

CsCl Purification of Plasmid DNA

Following analysis of pStep 4, one colony whose plasmid DNA showed the correct pattern upon digestion with restriction enzymes, was purified using a cesium chloride density gradient. The transformed *E. coli* cells were grown in 1 liter of L-broth containing ampicillin. The cells were harvested and the plasmid DNA extracted. The plasmid DNA was centrifuged in a cesium chloride/ethidium bromide solution to create a density gradient and separate plasmid DNA from RNA and any remaining cellular DNA and proteins. This purified plasmid DNA was used for sequence analysis and cassette mutagenesis.

Outline of the Strategy for Plastocyanin Gene Synthesis

Step 1

(a) Synthesize oligonucleotides 5, 6, 7 and 8.

(b) Kinase individual oligonucleotides and anneal 5 to 6, 7 to 8.

(c) Digest pBR322 with Eco RI and Ava I.

(d) Ligate annealed oligonucleotide duplexes into digested plasmid.

(e) Transform ligated plasmid into *E. coli*.

(f) Isolate plasmid DNA, pStep 1, and screen for insert.

Step 2

(a) Synthesize oligonucleotides 9, 10, 11 and 12.

(b) Kinase individual oligonucleotides and anneal 9 to 10, 11 to 12.

(c) Digest pStep 1 with Eco RI and Hind III.

(d) Ligate annealed oligonucleotide duplexes into digested plasmid.

(e) Transform ligated plasmid into *E. coli*.

(f) Isolate plasmid DNA, pStep 2, and screen for insert.

Step 3

(a) Synthesize oligonucleotides 13, 14, 15 and 16.

(b) Kinase individual oligonucleotides and anneal 13 to 14, 15 to 16.

(c) Digest pStep 2 with Ava I and Hind III.

(d) Ligate annealed oligonucleotide duplexes into digested plasmid.

(e) Transform ligated plasmid into *E. coli*.

(f) Isolate plasmid DNA, pStep 3, and screen for insert.

Step 4

(a) Synthesize oligonucleotides 17, 18, 19 and 20.

(b) Kinase individual oligonucleotides and anneal 17 to 18, 19 to 20.

(c) Digest pStep 3 with Nar I and Bgl II.

(d) Ligate annealed oligonucleotide duplexes into digested plasmid.

(e) Transform ligated plasmid into *E. coli*.

(f) Isolate plasmid DNA, pLasto, and screen for insert.

DNA Sequencing

Maxam-Gilbert Sequencing

The completed gene was initially sequenced using the Maxam-Gilbert chemical sequencing reactions (68). Although most of the bases could be assigned there were regions, especially runs of the same base, which were ambiguous as the exact number of bases in the sequence could not be assigned. At this point it was decided to proceed with attempts at expression of the protein from the synthetic gene (to be discussed in Chapter 2) while continuing to refine the DNA sequence in order to assign each base unambiguously.

Figure 12

Restriction digest for the second step of gene construction. 1.2% agarose gel stained with ethidium bromide. In step 2 Nae I and Ava I restriction sites were added to the plasmid. **Lane 1.** pBR322 (BRL) digested with Nae I. **Lane 2.** pBR322 (BRL) digested with Ava I. **Lane 3.** pBR322 (from a plasmid preparation) digested with Nae I. **Lane 4.** pBR322 digested with Ava I. **Lane 5.** pBR322. **Lane 6.** pStep 2 digested with Nae I. **Lane 7.** pStep 2 digested with Ava I. **Lane 8.** pStep 2. **Lane 9.** Colony 2 (another plasmid from step 2) digested with Nae I. **Lane 10.** Colony 2 digested with Ava I. **Lane 11.** Colony 2. **Lane 12.** Colony 3 digested with Nae I. **Lane 13.** Colony 3 digested with Ava I. **Lane 14.** Colony 3.

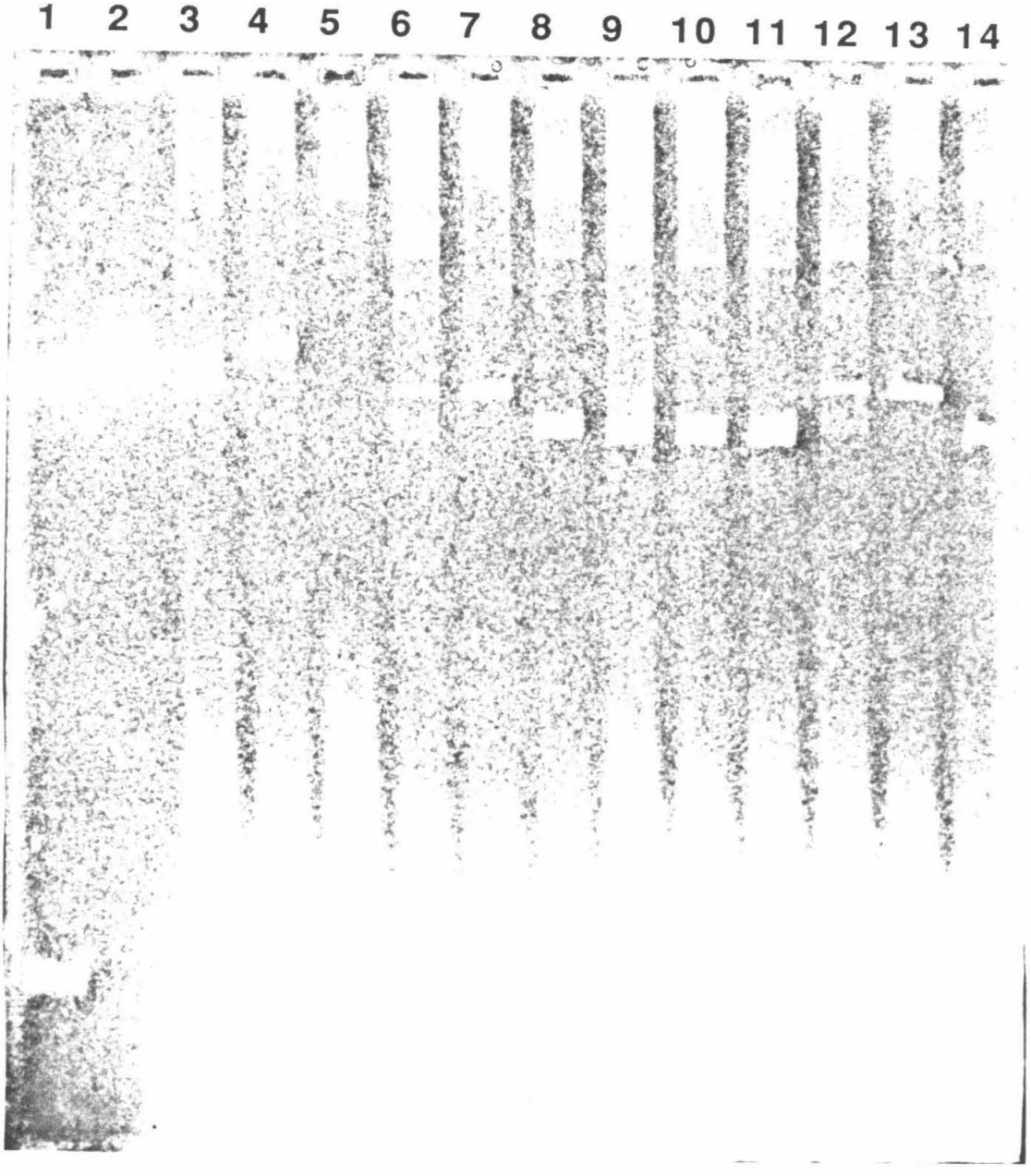

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 13

Restriction digest analysis for the third step of gene construction. 1.2% agarose gel stained with ethidium bromide. In step 3 Mlu I, Bgl II, Nar I and Hpa I restriction sites were added to the plasmid. Since there are no Bgl II or Mlu I sites in pBR322, lambda DNA was used as a standard. **Lane 1.** pBR322. **Lane 2.** pBR322 digested with Mlu I. **Lane 3.** pBR322 digested with Bgl II. **Lane 4.** λDNA. **Lane 5.** λDNA digested with Mlu I. **Lane 6.** λDNA digested with Bgl II. **Lane 7.** pStep 3. **Lane 8.** pStep 3 digested with Mlu I. **Lane 9.** pStep 3 digested with Bgl II. **Lane 10.** pStep 3 digested with Ava I. **Lane 11.** Colony 3 (another plasmid from step 3). **Lane 12.** Colony 3 digested with Mlu I. **Lane 13.** Colony 3 digested with Bgl II. **Lane 14.** Colony 3 digested with Ava I.

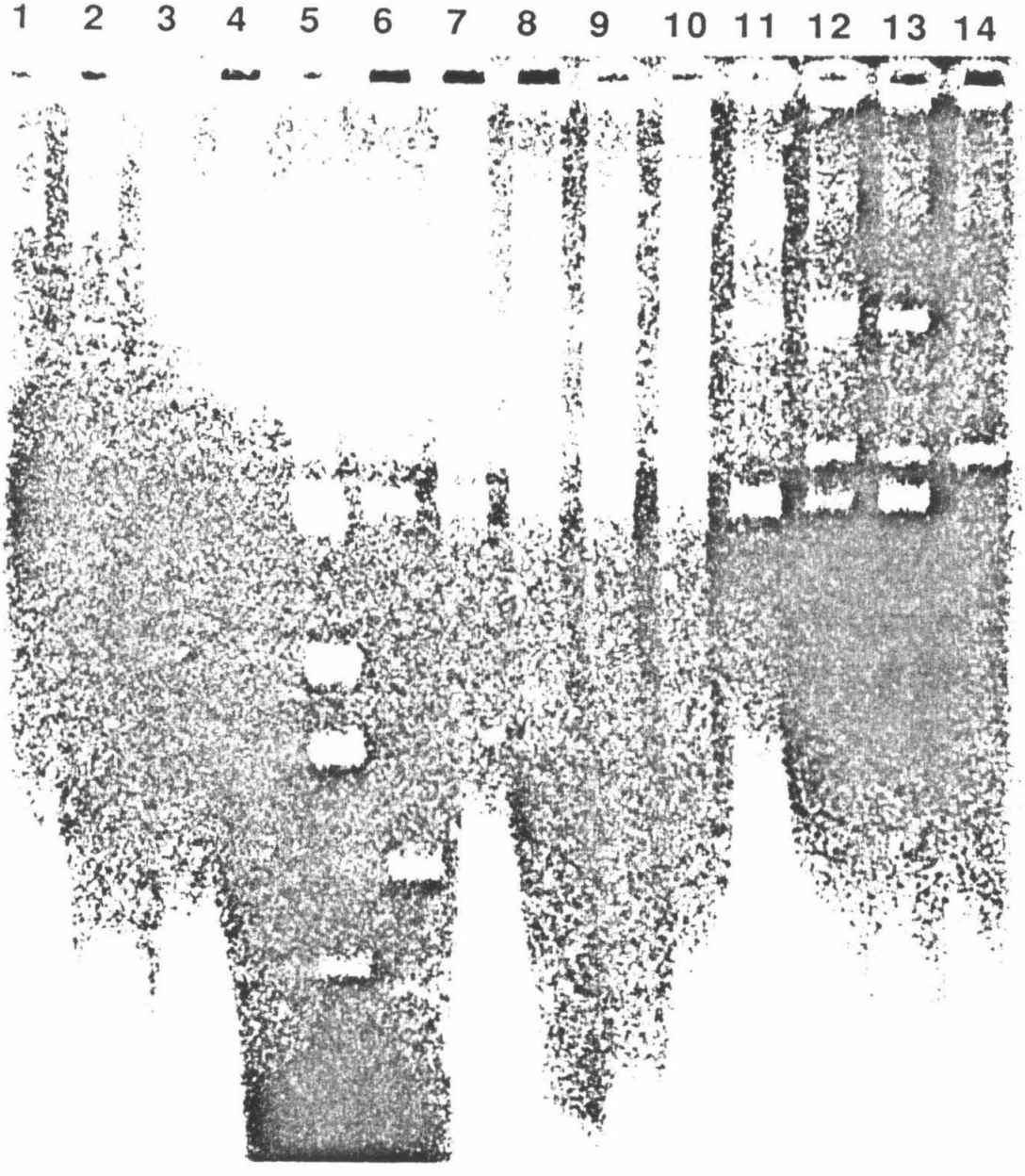

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 14

Restriction digest analysis for the final step of gene construction. 1.2% agarose gel stained with ethidium bromide. No new restriction sites were added to the plasmid in this step therefore analysis was based on a size increase of the gene fragment. Plasmid without the final insert (pStep 3) gives fragments of 943 and 2184 base pairs when digested with Pst I and Hind III. Plasmid with the final insert would give fragments of 1043 and 2184 base pairs. **Lane 1.** Colony A (plasmid from step 4) digested with Pst 1 and Hind III. **Lane 2.** Colony B digested with Pst I and Hind III. **Lane 3.** Colony C digested with Pst I and Hind III. **Lane 4.** Colony D digested with Pst I and Hind III. **Lane 5.** Colony E digested with Pst I and Hind III. **Lane 6.** Colony F (pLasto 1) digested with Pst I and Hind III. **Lane 7.** pStep 3 digested with Pst I and Hind III. **Lane 8.** pBR322 digested with Eco RI, Ava I and Pst I to give fragments of 752, 1425 and 2184 base pairs. **Lane 9.** pStep 3 digested with Nar I as a linear standard. **Lane 10.** Colony B digested with Nar I. **Lane 11.** Colony B. **Lane 12.** Colony F (pLasto) digested with Nar I. **Lane 13.** Colony F (pLasto). **Lane 14.** Colony C digested with Nar I.

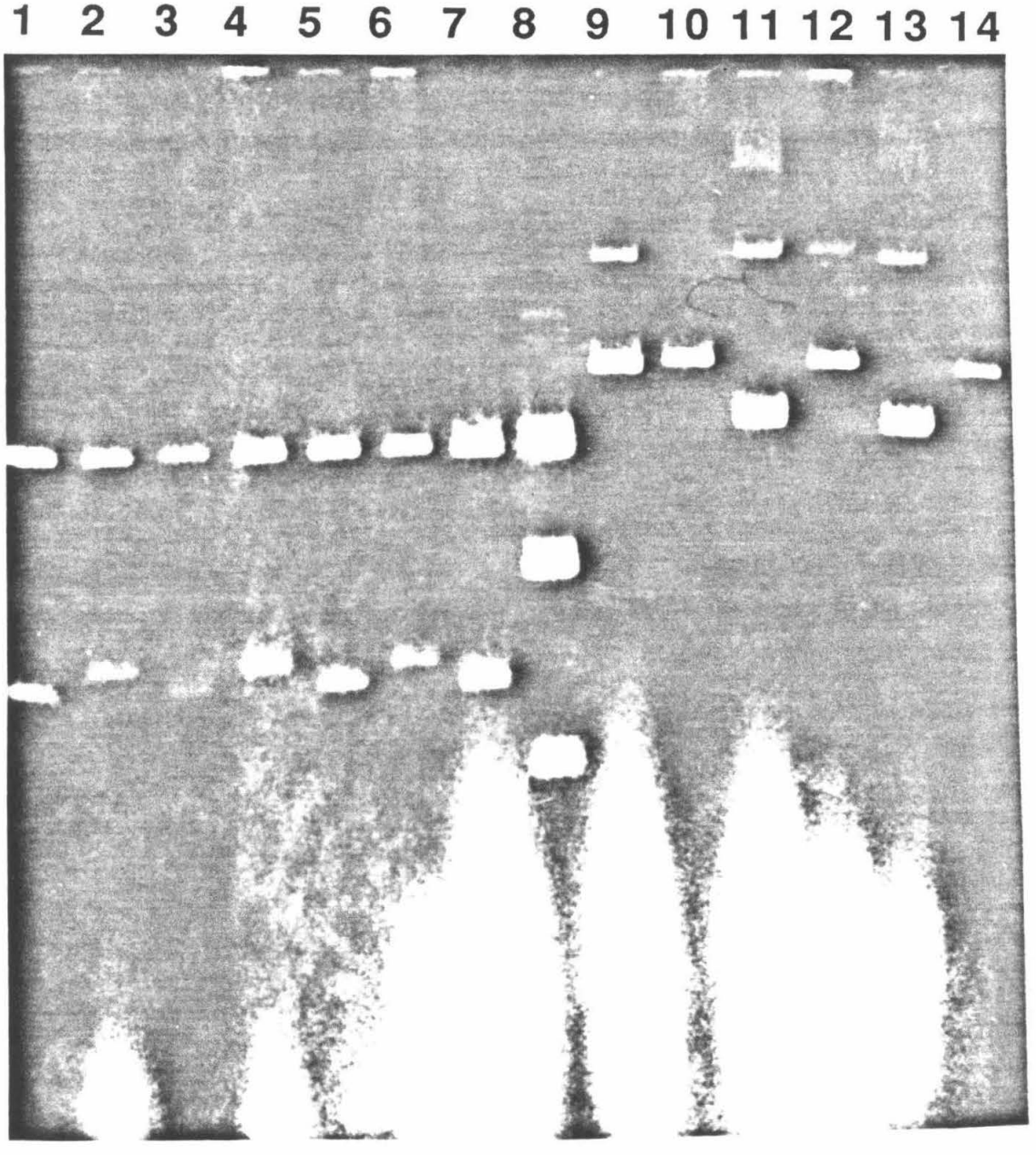
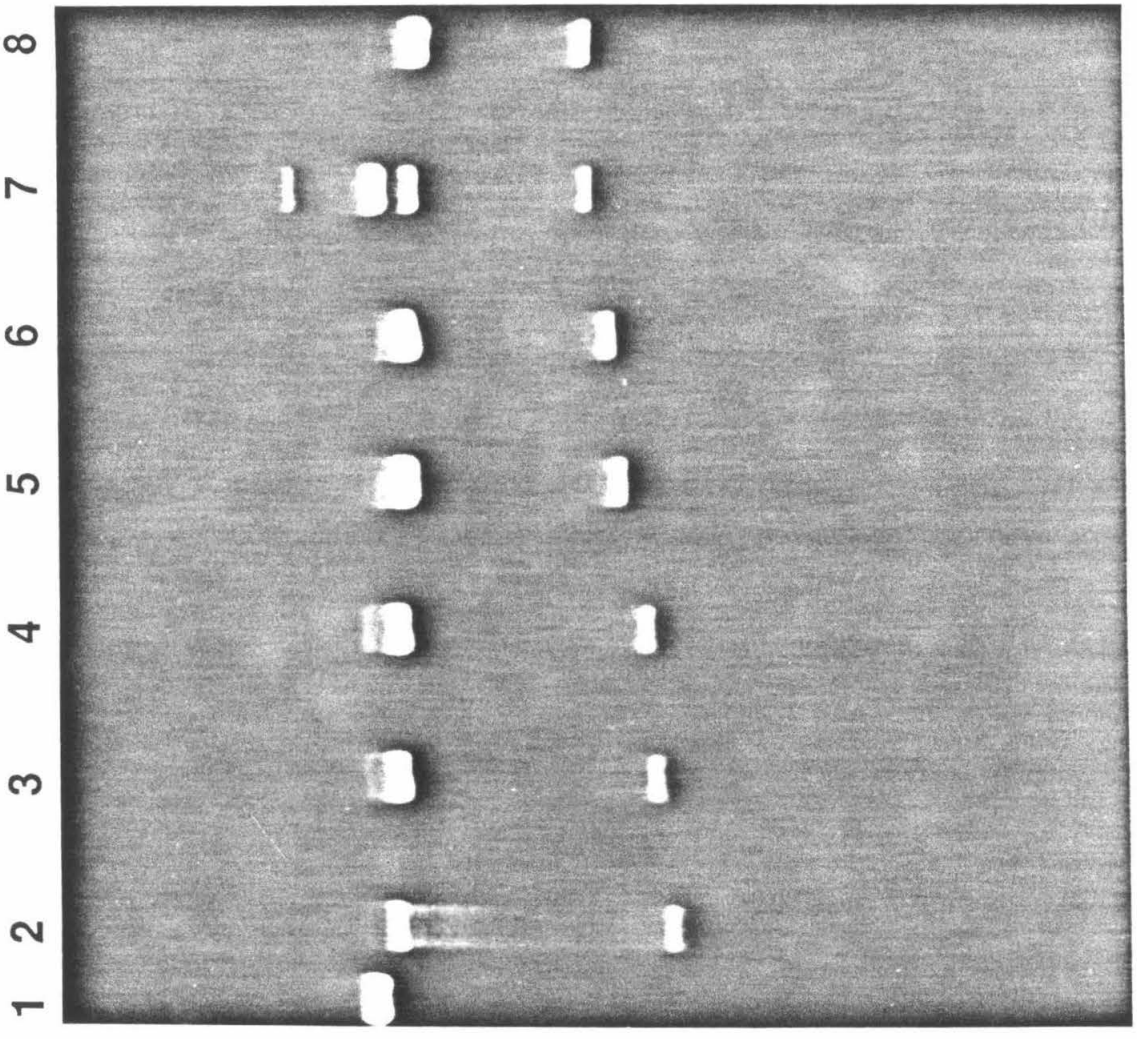

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 15

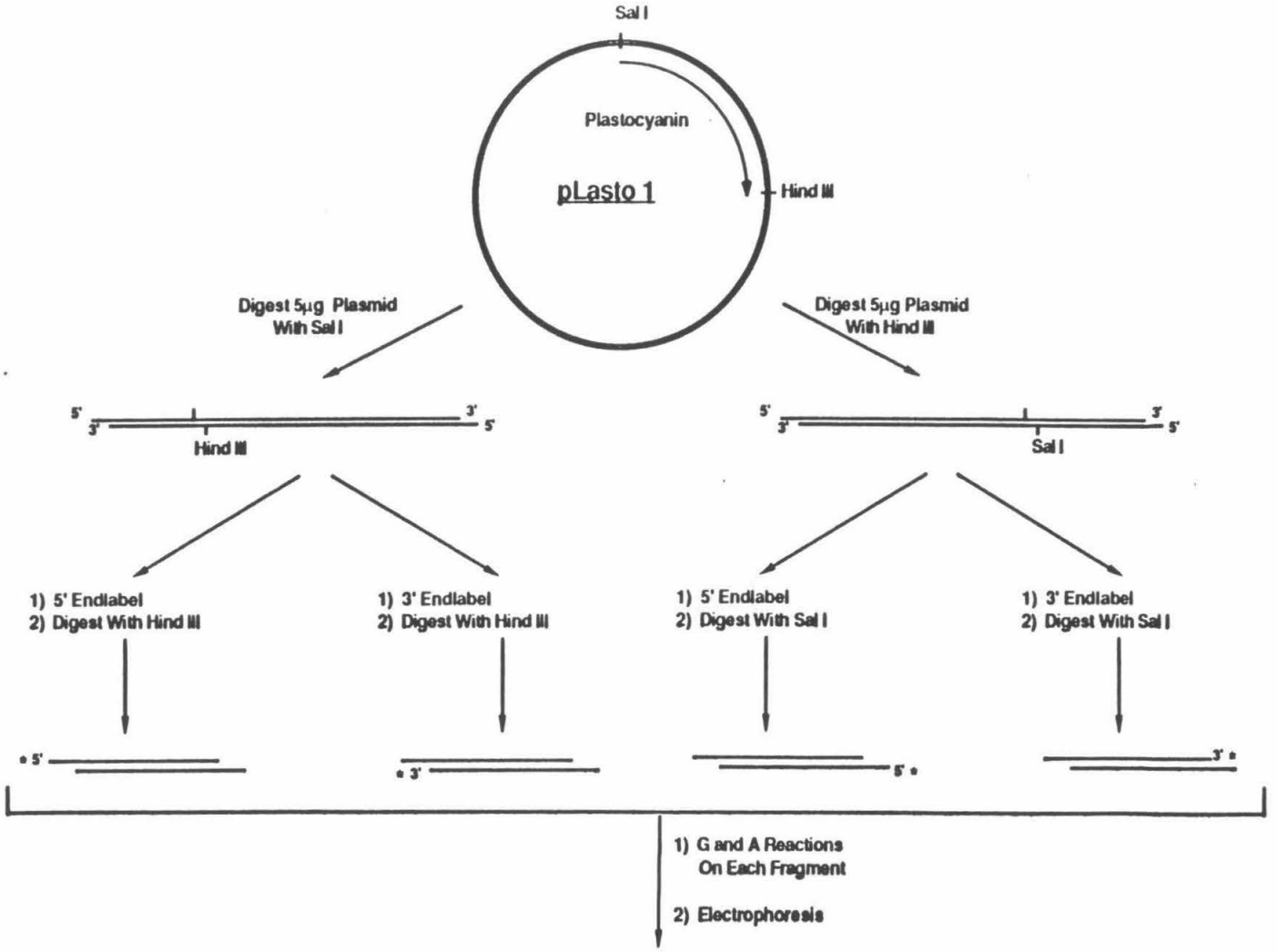
Restriction digest analysis of pLasto 1 following CsCl purification of the DNA. 1.2% agarose gel stained with ethidium bromide. The reactions were loaded onto the gel in order to verify the location of each restriction site within the plastocyanin gene. This experiment shows the importance of DNA sequencing as no mutations were apparent from this restriction digest analysis. **Lane 1.** pLasto 1 digested with Pst I. **Lane 2.** pLasto 1 digested with Pst I and Sal I. **Lane 3.** pLasto 1 digested with Pst I and Bam HI. **Lane 4.** pLasto 1 digested with Pst I and Eco RI. **Lane 5.** pLasto 1 digested with Pst I and Ava I. **Lane 6.** pLasto 1 digested with Pst I and Bgl II. **Lane 7.** pLasto 1 digested with Pst I and Nar I. **Lane 8.** pLasto 1 digested with Pst I and Hind III.


1
2
3
4
5
6
7
8

DNA Sequencing Using an A Specific Reaction

The chemical sequencing strategy was revised with the advent of a new chemical sequencing reaction which afforded cleavage at adenine residues exclusively (69). Using this A reaction along with the Maxam-Gilbert G reaction, which cleaves only at guanine residues, each base in the gene can be identified by a single band on a polyacrylamide gel. This strategy avoids the A + G and C + T reaction (which were sometimes ambiguous in our hands), of the Maxam-Gilbert sequencing strategy and the DNA cloning steps involved with Sanger dideoxy sequencing (70). The strategy for sequencing the gene for plastocyanin is outlined in Figure 16.

The plasmid pLasto 1 was transformed into *E. coli* HB101. A one liter culture was then grown at 37°C and the plasmid amplified with chloramphenicol. The cells were harvested and the plasmid isolated and purified using cesium chloride density gradient centrifugation (71). Fifteen μg of the purified pLasto 1 was digested with Hind III and another 15 μg was digested with Sal I. The linearized plasmids were isolated on an agarose gel to remove any residual RNA which may interfere with the 5′ end labelling reactions. The isolated linearized plasmids were then divided in half for the labelling reactions. Half of each reaction (Sal I digest and Hind III digest) was subjected to treatment with calf alkaline phosphatase to remove the 5′ terminal phosphates. The reaction was stopped by phenol extraction and ethanol precipitation. The DNA was labelled with γ^{32}P ATP at the 5′ end using T4 polynucleotide kinase. The other half of the DNA from the restriction digests was labelled at the 3′ end using the large fragment of DNA polymerase I Klenow to fill in the α^{32}P-nucleotide. Non-radioactive nucleotides were also added to the reaction in order to fill in the duplex completely to the end. All of the labelled DNA was then digested with a



DNA Sequencing protocol using G and A specific reactions

Figure 16

second enzyme; Sal I linearized plasmid was digested with Hind III and Hind III linearized plasmid was digested with Sal I, and the smaller ~300 base pair fragments were isolated from an agarose gel. These fragments contained the plastocyanin gene singly labelled at one end. Each of these isolated labelled fragments was ethanol precipitated and the total radioactivity was estimated. The samples were resuspended in 0.25 mM Tris-HCl pH 7.5 to give similar concentrations of radioactivity in each sample. Cleavage reactions were performed on approximately 5 mR/hr of radioactive DNA. Cleavage at guanine was achieved using dimethyl sulfate as described by Maxam and Gilbert (68). Cleavage at adenine was achieved using K_2PdCl_4 as described in a recently published reaction by B. L. Iverson (69). Both reactions were followed by treatment with piperidine at 90°C, lyophilized and resuspended in formamide loading buffer. The samples were loaded onto an 8% denaturing polyacrylamide gel. The gel was dried, autoradiographed and the sequence was examined. Recall that plasmid pLasto 1 is the original construction of the plastocyanin gene in pBR322. The sequencing gel showed an extra thymine (T) at Phe 70 of the protein sequence as shown in Figures 17 and 18. This created a termination codon at position 71 in the protein sequence. Since three of the four amino acids involved in copper binding occur after this position the protein could not possibly be functional. The reactions were also loaded onto a 20% polyacrylamide gel which was autoradiographed and analyzed. This higher percentage gel verified the sequence into each labelled restriction site.

Cassette Mutagenesis

5′ End Labelling and Annealing of Oligonucleotides

Since the gene was designed for cassette mutagenesis, oligonucleotides were synthesized to span the region from the Bgl II and Nar I restriction sites.

Figure 17

DNA sequence analysis of pLasto 1. Autoradiogram of a standard 8% sequencing gel. The DNA fragments were isolated and labelled according to procedures described in the experimental section. Cleavage reactions specific for guanine (G) and adenine (A) were used to determine the sequence. The same DNA fragment was radioactively labelled with ^{32}P at the 5′ or 3′ end, therefore G and A reactions give a cleavage band for every base. The same reactions were loaded onto the gel at three separate times in order to increase the number of bases visible on the gel. The additional thymine (T) is indicated by an arrow in the first lanes of the Hind III labelled fragment. The region where a second mutation was found in a different plasmid (a deletion of a T) is also indicated in order to show that this sequence was correct in this original construction.

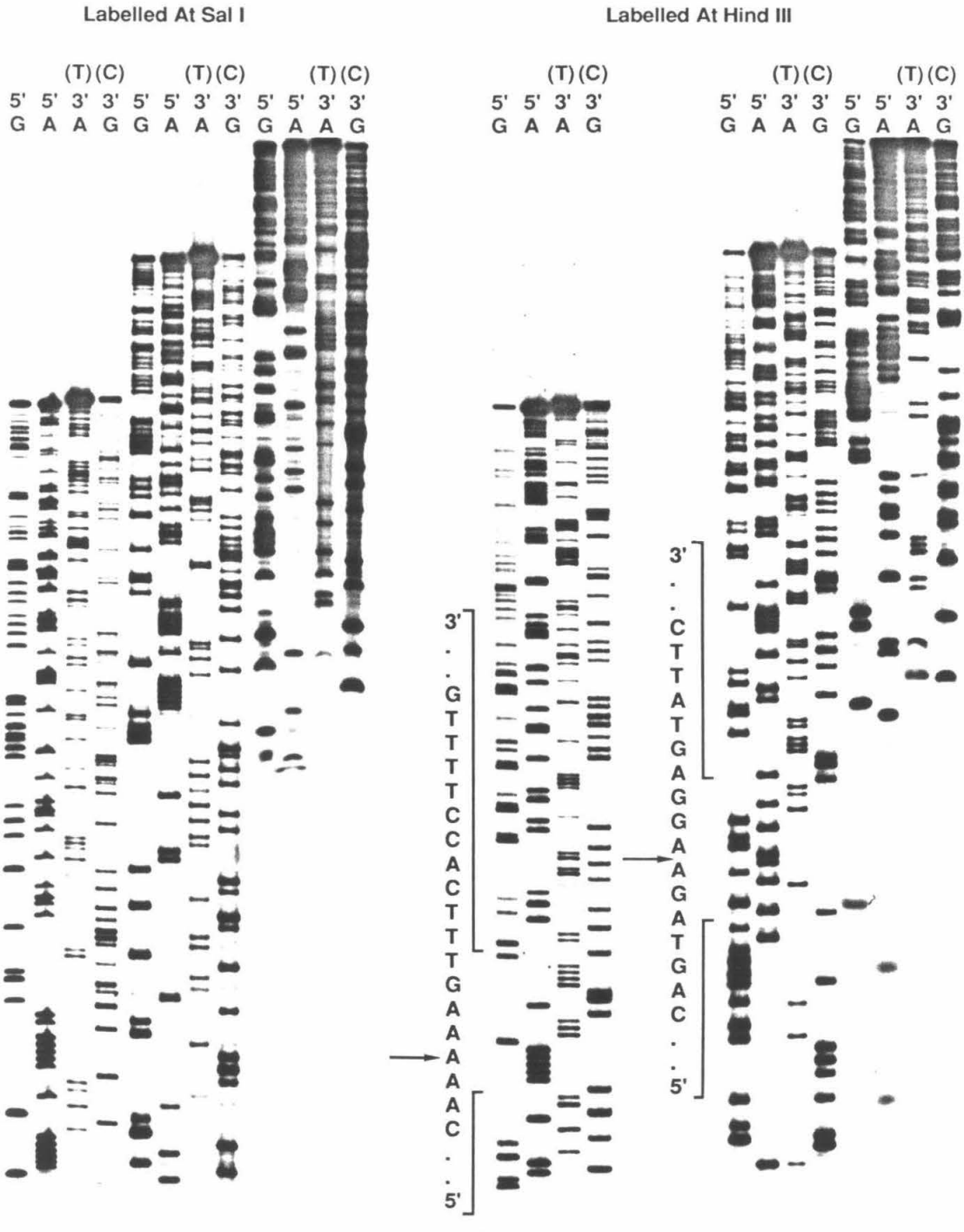
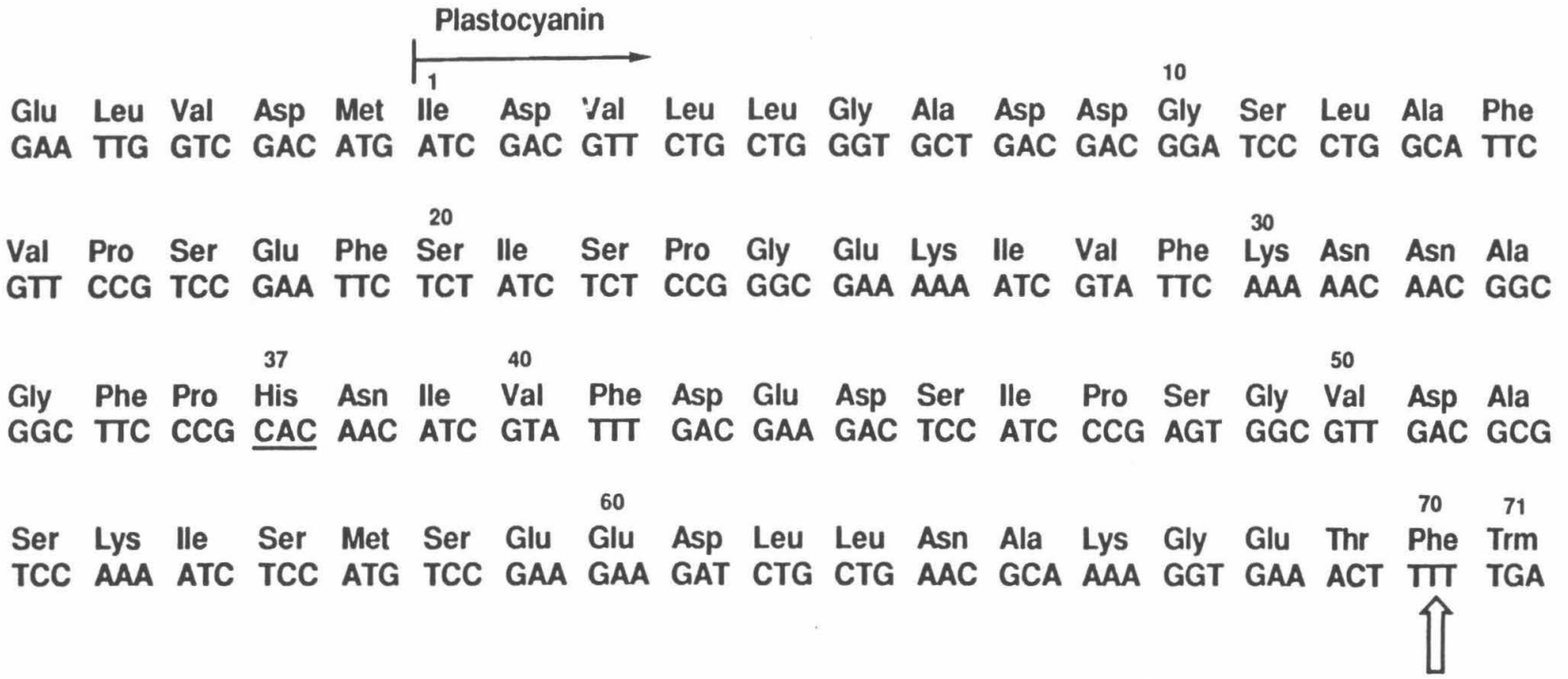

Labelled At Sal I
Labelled At Hind III
(T) (C)
5' G
5' A
3' A
3' G
3' A
3' A
3' G
3' G
3' . . G T T T T C C A C T T T G A A A A A C . . 5'
3' . . C T T A T G A G G A A G A T G A C . . 5'

Figure 18

DNA sequence and translation of the plastocyanin gene in pLasto 1 which shows the result of the additional thymine (T) on the protein sequence as indicated by the arrow. This mutation of the DNA creates a stop codon at position 71 in the protein sequence which terminates the protein prior to the occurrence of three of the four amino acids involved in binding of the copper atom.


Plastocyanin
1
10
Glu Leu Val Asp Met Ile Asp Val Leu Leu Gly Ala Asp Asp Gly Ser Leu Ala Phe
GAA TTG GTC GAC ATG ATC GAC GTT CTG CTG GGT GCT GAC GAC GGA TCC CTG GCA TTC
20
30
Val Pro Ser Glu Phe Ser Ile Ser Pro Gly Glu Lys Ile Val Phe Lys Asn Asn Ala
GTT CCG TCC GAA TTC TCT ATC TCT CCG GGC GAA AAA ATC GTA TTC AAA AAC AAC GGC
37
40
50
Gly Phe Pro His Asn Ile Val Phe Asp Glu Asp Ser Ile Pro Ser Gly Val Asp Ala
GGC TTC CCG CAC AAC ATC GTA TTT GAC GAA GAC TCC ATC CCG AGT GGC GTT GAC GCG
60
70
71
Ser Lys Ile Ser Met Ser Glu Glu Asp Leu Leu Asn Ala Lys Gly Glu Thr Phe Trm
TCC AAA ATC TCC ATG TCC GAA GAA GAT CTG CTG AAC GCA AAA GGT GAA ACT TTT TGA

The oligonucleotides were purified on a 15% denaturing polyacrylamide gel; the bands were visualized using a TLC plate under the gel and illuminating with UV light. The bands were excised, crushed and the DNA eluted with 0.2 M NaCl (72). The purified oligonucleotides were then dialyzed against 0.25 mM Tris·HCl, pH 7.5. The concentration and purity of DNA was then determined by UV absorbance at 260 and 280 nm.

Vector Preparation and Ligation

Purified plasmid pLasto was digested with Nar I, which does not digest completely, so the reaction mixture was loaded onto a 1.2% agarose gel. The band of linear DNA, visualized by ethidium bromide fluorescence, was excised and the DNA electroeluted, followed by ethanol precipitation. The pellet was redissolved in 0.25 mM Tris·HCl, pH 7.5, and digested with Bgl II, ethanol precipitated and half of the DNA was treated with calf alkaline phosphatase. The reaction mixture was loaded onto a 1.2% agarose gel and the largest linear DNA band was purified as described above. The concentration and purity of DNA was determined by the UV absorbance at 260 and 280 nm. The spectrum was analyzed from 200 to 300 nm in order to analyze the shape of the curve generated by the DNA. After the concentration of the DNA was determined the mixture was lyophilized to an acceptable volume for further manipulation. The purified oligonucleotides were phosphorylated at their 5′ ends with T4 polynucleotide kinase and ATP. Complementary oligonucleotides were then annealed together by heating at 90°C for 3 minutes and cooling slowly to room temperature to create two oligonucleotide duplex structures. These duplexes were ligated into the purified digested plasmid using T4 DNA ligase in a reaction which proceeded at 15°C for 12 hours. Two ligation reactions were tried, one where the digested plasmid was treated with calf alkaline phosphatase after digestion, and a second where it was not. The

ligation mixtures were transformed into *E. coli* HB101 and the cells were plated onto L-agar containing ampicillin at a concentration of 50 mg/L. The plate of cells transformed with ligation mixture A (phosphatased vector) gave only 2 colonies, referred to as 6-7, after growth at 30°C overnight while ligation mixture B (no phosphatase treatment) gave 5 colonies, referred to as 1-5.

CsCl Purification of Plasmid DNA

All seven of these colonies were grown in 2 mls of L-broth with amipicillin. The cells were grown at 37°C for 12 hours, at this time the cells were harvested and the plasmid DNA isolated and analyzed using restriction enzymes to confirm the presence of the plastocyanin gene. Five of the seven plasmids gave consistent results upon analysis with restriction enzymes and were pursued further. These colonies were grown in 500 mls of L-broth with ampicillin and the plasmid DNA amplified with chloramphenicol. After growth at 37°C the cells were harvested the plasmid DNA isolated and purified by two subsequent cesium chloride density gradient centrifugations. The purified DNA was then dialyzed against three 3 liter changes of 0.25 mM Tris, pH 7.5. Following dialysis the concentration and purity of the DNA was analyzed by UV absorbance at 260 and 280 nm.

DNA Sequencing

5 μg of each plasmid 2, 3, 5, 6, 7 was digested with Hind III and the linear band isolated on a 1.2% agarose gel. The band was excised from the gel, the DNA electroeluted and ethanol precipitated. The pellet was redissolved in the appropriate buffer and a calf alkaline phosphatase reaction performed to remove the 5′ terminal phosphates; the reaction was stopped by phenol extraction and ethanol precipitation. The pellet was redissolved in buffer for a kinase reaction which was initiated by the addition of T4 polynucleotide

kinase and $\gamma^{32}P$ ATP; the labelled DNA was ethanol precipitated and redissolved in buffer for a Sal I digest. The reaction mixture was loaded onto a 1.2% agarose gel which was run only until the 300 base pair band containing the gene was just separated from the rest of the plasmid. This allows most of the unincorporated radioactive nucleotides to remain on the gel and avoids excess contamination of a large buffer volume. The labelled DNA band was visualized by ethidium bromide fluorescence, excised from the gel, electroeluted and ethanol precipitated. Each pellet was dissolved in 0.25 mM Tris, pH 7.5, and the sample was divided in half. Maxam-Gilbert G reactions and K_2PdCl_4 A reactions were performed as described previously. Equal amounts of radioactivity were loaded onto each lane of an 8% denaturing polyacrylamide gel and autoradiographed.

Since only one particular region of the gene was being examined at this time each plasmid was only labelled at one end (Hind III) and the gel was loaded twice with each sample. This only allowed samples 2, 3, 5 and 6 to be analyzed on this first gel. When the results were analyzed in the region of the initial mutation 2, 3 and 5 were found to contain the extra T as seen in pLasto 1. Recall these plasmids came from the ligation where calf alkaline phosphatase was not used on the digested pLasto 1 prior to the ligation reaction, and are most likely a religation of the initial plasmid. Plasmid 6 did not contain the extra T in the region of interest and was pursued further. Plasmid 6 was labelled 5′ and 3′ individually at the Sal I and Hind III sites as previously described. G and A reactions were performed on all four labelled fragments. The reactions were loaded onto an 8% denaturing polyacrylamide gel along with the G and A reactions of plasmid 7 labelled at the 5′ end of the Hind III site as described. The gel was autoradiographed and the results examined as shown in Figures 19-21. In plasmid 6 as discussed previously the

Figure 19

Sequence analysis of plasmid 6. Autoradiogram of a standard 8% polyacrylamide sequencing gel. The DNA fragments were isolated and labelled according to procedures described in the experimental section. Cleavage reactions specific for guanine (G) and adenine (A) were used to determine the sequence after the DNA fragments were radioactively labelled with ^{32}P at the 5′ or 3′ end. The region where the original mutation occurred, an addition of a thymine (T) is indicated by an arrow and was corrected as shown in the first lanes labelled at Hind III. The second mutation, a deletion of a thymine (T) is indicated by an arrow in the second lanes of DNA labelled at Hind III.

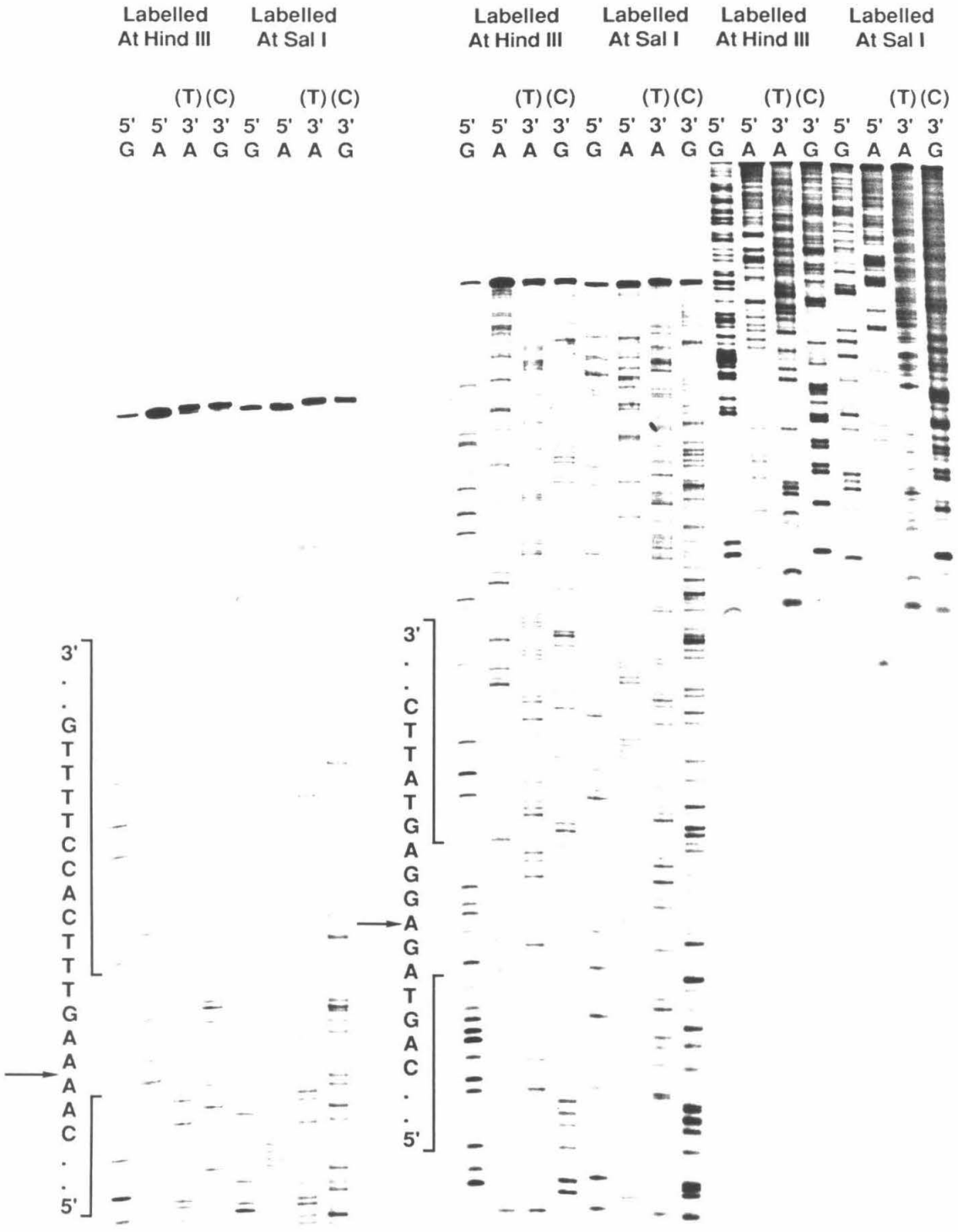

Labelled
At Hind III
Labelled
At Sal I
Labelled
At Hind III
Labelled
At Sal I
Labelled
At Hind III
Labelled
At Sal I
(T) (C)
5' G
5' A
3' A
3' G
5' G
5' A
3' A
3' G
3' . . G T T T T C C A C T T T T G A A A A C . . 5'
3' . . C T T A T G A G G A G A A T G A C . . 5'

Figure 20

DNA sequence and translation of plasmid 6, showing the result of a thymine (T) deletion at Phenylalanine (Phe) 82 which becomes a Serine as indicated by the underlined sequence (numbering begins at the first Ile of the plastocyanin gene). Although a termination codon is not created until position 96, three of the four amino acids involved in binding of the copper occur after position 82 and are destroyed by the altered sequence following the mutation.

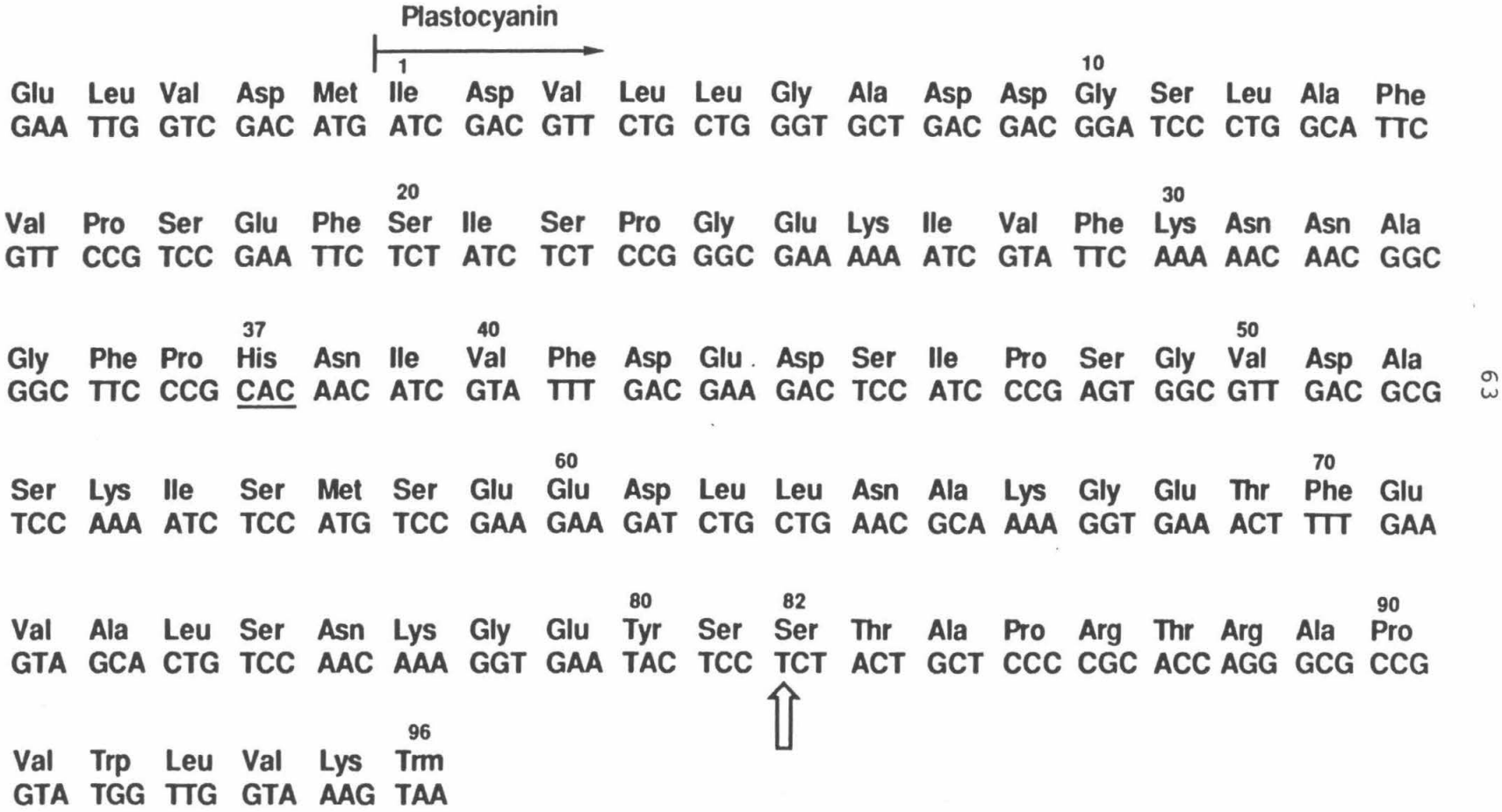
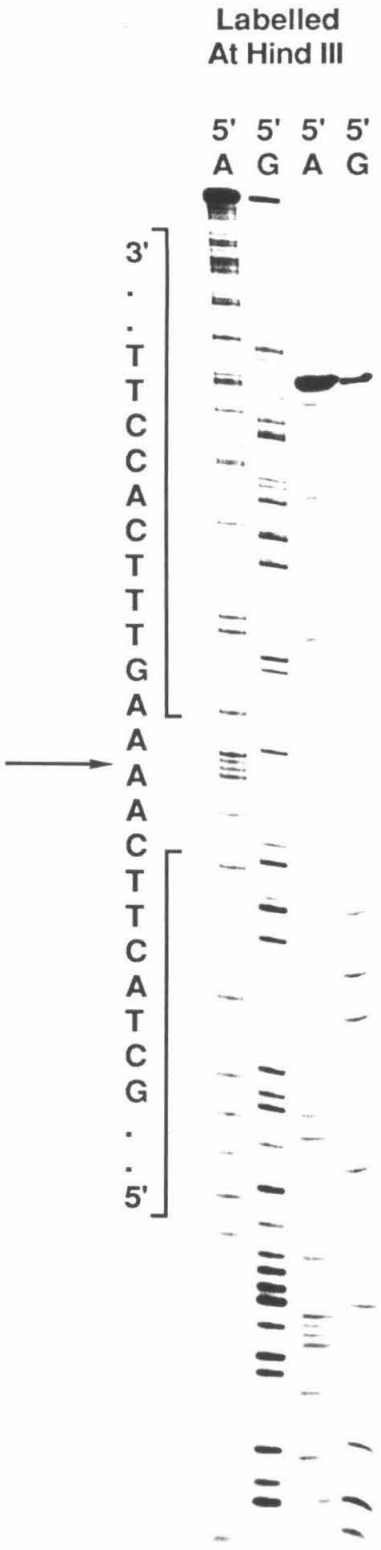

Plastocyanin
1
10
Glu Leu Val Asp Met Ile Asp Val Leu Leu Gly Ala Asp Asp Gly Ser Leu Ala Phe
GAA TTG GTC GAC ATG ATC GAC GTT CTG CTG GGT GCT GAC GAC GGA TCC CTG GCA TTC
20
30
Val Pro Ser Glu Phe Ser Ile Ser Pro Gly Glu Lys Ile Val Phe Lys Asn Asn Ala
GTT CCG TCC GAA TTC TCT ATC TCT CCG GGC GAA AAA ATC GTA TTC AAA AAC AAC GGC
37
40
50
Gly Phe Pro His Asn Ile Val Phe Asp Glu Asp Ser Ile Pro Ser Gly Val Asp Ala
GGC TTC CCG CAC AAC ATC GTA TTT GAC GAA GAC TCC ATC CCG AGT GGC GTT GAC GCG
60
70
Ser Lys Ile Ser Met Ser Glu Glu Asp Leu Leu Asn Ala Lys Gly Glu Thr Phe Glu
TCC AAA ATC TCC ATG TCC GAA GAA GAT CTG CTG AAC GCA AAA GGT GAA ACT TTT GAA
80
82
90
Val Ala Leu Ser Asn Lys Gly Glu Tyr Ser Ser Thr Ala Pro Arg Thr Arg Ala Pro
GTA GCA CTG TCC AAC AAA GGT GAA TAC TCC TCT ACT GCT CCC CGC ACC AGG GCG CCG
96
Val Trp Leu Val Lys Trm
GTA TGG TTG GTA AAG TAA

Figure 21

DNA sequence analysis of plasmid 7 (pLasto 2) in the region of the original mutation, an addition of thymine (T). Autoradiogram of a standard 8% polyacrylamide sequencing gel. The DNA fragments were isolated and labelled according to procedures described in the experimental section. Cleavage reactions specific for guanine (G) and adenine (A) were used to determine the sequence following the radioactive labelling of the DNA fragments with ^{32}P at the 5′ or 3′ end. The original mutation of five thymines (T) in a row has been corrected to four thymines in plasmid 7 as indicated by the arrow.


Labelled
At Hind III
5' A
5' G
5' A
5' G
3'
T
T
C
C
A
C
T
T
T
T
G
A
A
A
A
A
C
T
T
C
A
T
C
G
5'

original mutation, an extra T at Phe 75 in the protein sequence, had been corrected but a second mutation, a deletion, was found at Phe 87. This mutation was a deletion of a T in the DNA sequence which altered the DNA sequence after this position and resulted in a termination codon 16 amino acids after the mutation. The deletion precedes three of the four amino acids which bind copper in plastocyanin so again the protein would not be functional.

Plasmid 7 showed the correct sequence in the region of the original mutation (no addition of a T) and was sequenced further to check for additional mutations. The DNA was individually labelled 5′ and 3′ at the Sal I and Hind III sites as described earlier. DNA cleavage reactions specific for G and A were performed on each of the four labelled DNA fragments; the reactions were loaded onto an 8% polyacrylamide gel and autoradiographed. The sequence was found to be correct although the sequence could only be clearly read to within 20 bases of each labelled restriction site. In order to read this portion of the sequence the same reactions were loaded onto a 20% denaturing polyacrylamide gel. The sequence could now be clearly read all the way into the bases of the labelled restriction site, and the entire sequence was now confirmed to be correct. The two sequencing gels are shown in Figures 22 and 23. Due to the shorter fragment length of DNA analyzed on the 20% gel, the difference in mobility of the 5′ and 3′ labelled fragments became apparent. The basis for this mobility difference can be explained by a difference in charge as shown in Figure 24. The fragment labelled at the 3′ end has fewer negative charges than a fragment labelled at the 5′ end thus creating a difference in mobility when the fragment is short enough. This occurs when the fragment length is approximately 6-8 base pairs; at this point the percentage difference in charge between the two labelled fragments is

Figure 22

Sequence analysis of pLasto 2 (plasmid 7) to verify the entire DNA sequence. Autoradiogram of a standard 8% polyacrylamide gel. The DNA fragments were isolated and radioactively labelled with ^{32}P according to procedures described in the experimental section. Cleavage reactions specific for guanine (G) and adenine (A) were used to determine the sequence following the radioactive labelling of the DNA fragments with ^{32}P at the 5′ or 3′ end. There were no mutations in the plastocyanin gene from this plasmid. The regions where two previous mutations were found are indicated with arrows and are shown to be correct.

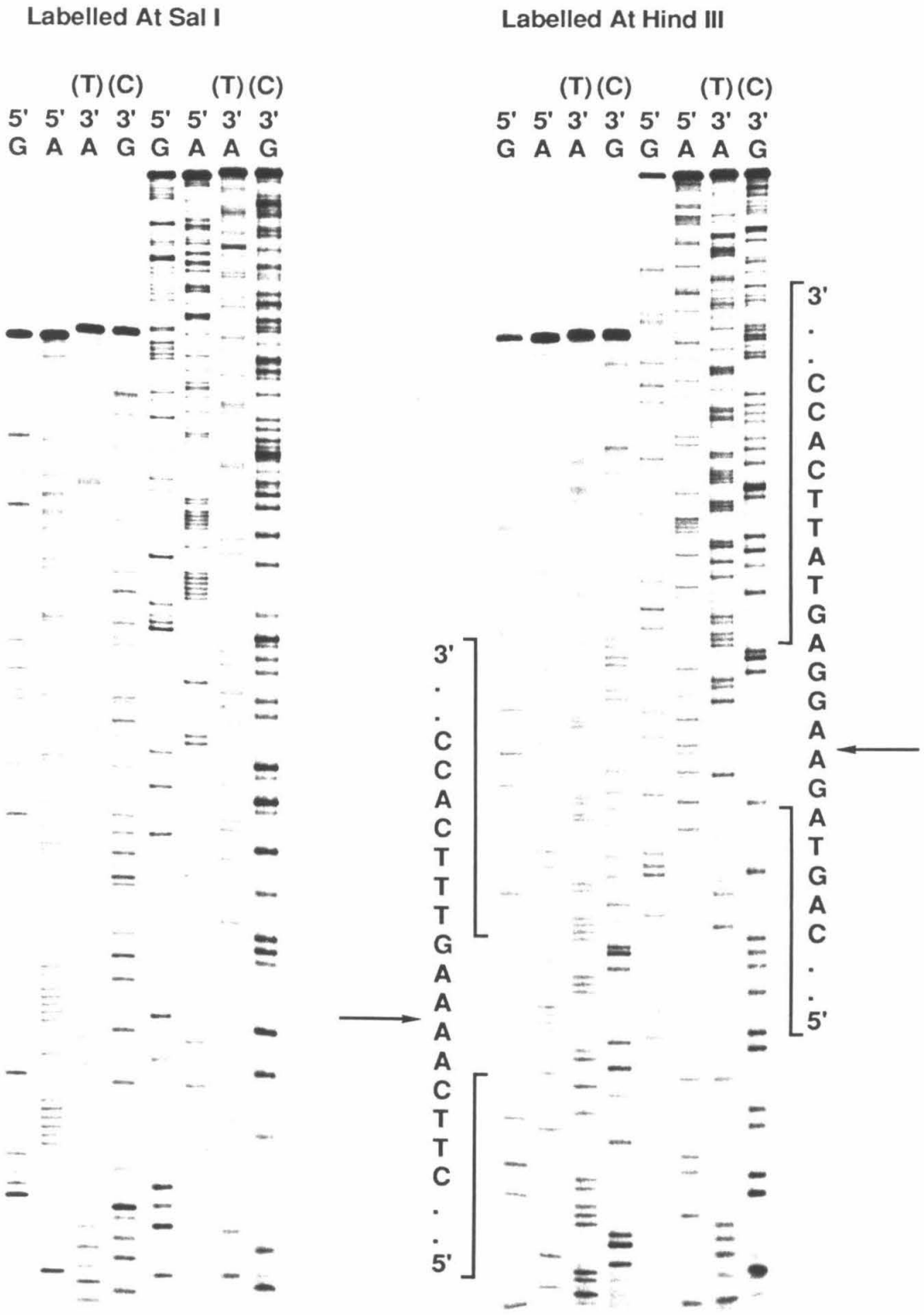

Labelled At Sal I
Labelled At Hind III
(T) (C) (T) (C)
5' 5' 3' 3' 5' 5' 3' 3'
G A A G G A A G
3' . . C C A C T T T G A A A A C T T C . . 5'
3' . . C C A C T T A T G A G G A A G A T G A C . . 5'

Figure 23

Sequence analysis of pLasto 2 (plasmid 7). Autoradiogram of a 20% denaturing polyacrylamide gel. The same reaction mixtures were used as described for Figure 22. The 20% gel allows bases to be read into the radioactively labelled restriction site. Due to a slight change in mobility of these short fragments (see Figure 24) it is important to verify the sequence at a restriction site in order to read the bases correctly from a 20% gel. The sequence on the gel reads as indicated, however, the actual sequence labelled at Hind III is ATTGGCAATTGATCTTCGAAT. The underlined T is verified by the Hind III site (overlined) and is used as a base from which to read the rest of the DNA sequence. Similarly, from the Sal I labelled end the sequence is actually GGTCGACATGATCGTCGTTCTGCTGGG. The underlined bases are verified by the Sal I site (overlined) and are used as a base to orient the rest of the sequence on the gel.

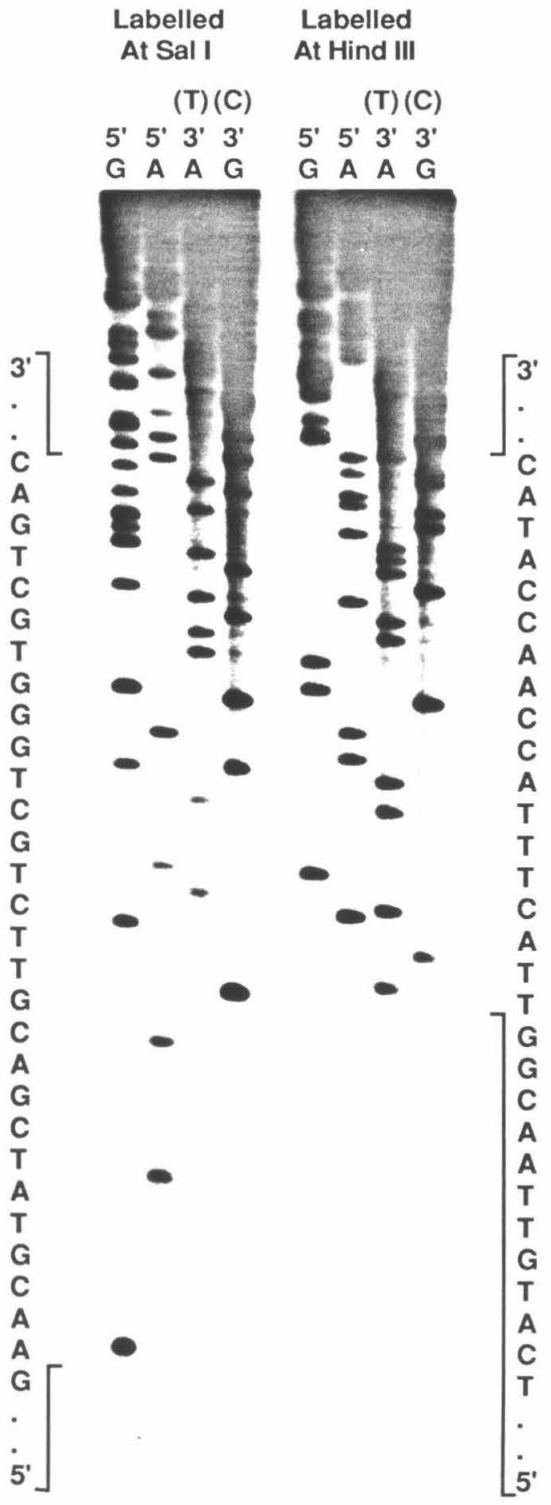
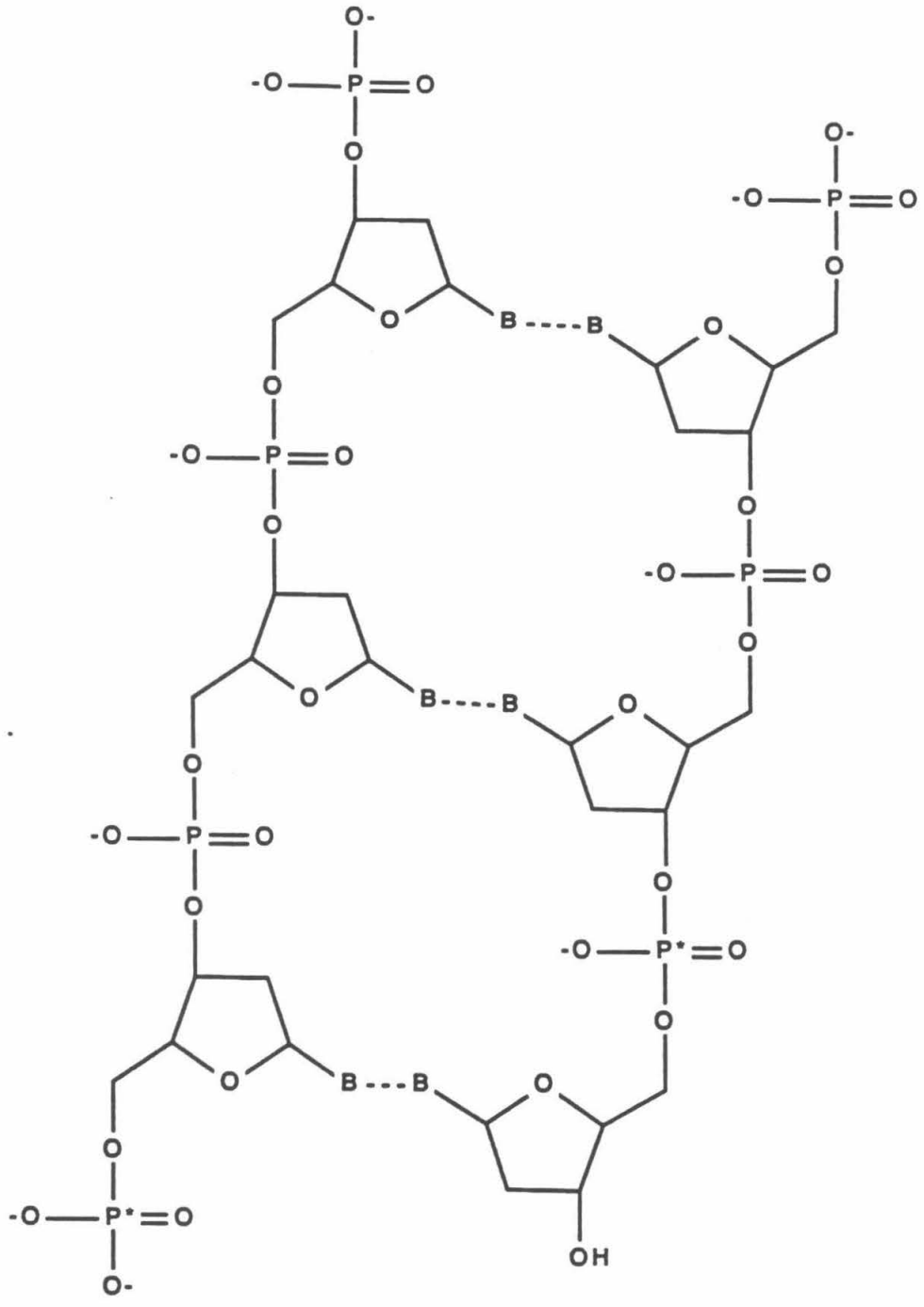

Labelled
At Sal I
Labelled
At Hind III
(T) (C)
5' 5' 3' 3'
G A A G
3'
. .
C A G T C G T G G G T C G T C T T G C A G C T A T G C A A G
. .
5'
3'
. .
C A T A C C A A C C A T T T C A T T G G C A A T T G T A C T
. .
5'

Figure 24

Illustration of DNA fragments radioactively labelled with ^{32}P at 5′ and 3′ positions. This figure shows the difference in charge created by the end labelling, which causes the difference in mobility observed between the shorter radioactively labelled fragments on a 20% sequencing gel. This difference in mobility is only apparent when very short fragments of DNA are analyzed.



5' Endlabel **3' Endlabel**

6 Negative Charges **4 Negative Charges**

enough to cause a significant change in mobility. Fortunately, the shortest fragments occur within restriction sites which are known to be correct by the complete cutting of the DNA at these sites, this allows the sequence to be confirmed at these sites and since the 3′ end labelled fragment will be expected to run slower on the gel, the sequence can still be easily read from the gel under these conditions. This gene could now be used in expression vectors in order to express the protein plastocyanin; these experiments will be discussed in Chapter 2. This plasmid which contains the correct DNA sequence for the plastocyanin gene will be known hereafter as pLasto 2, the DNA sequence with restriction sites and translation of pLasto 2 is shown in Figure 25.

Mutation Analysis

Search for Promoter Elements

Due to the frequency of mutations observed in the synthetic gene the possibility that plastocyanin was having a lethal effect on *E. coli* was considered. In order for this to be true the protein must be expressed in *E. coli* after transformation. Although the promoter and the gene for tetracycline were removed during the construction of the gene for plastocyanin in pBR322, sequences which are homologous to the consensus sequence of *E. coli* promoters are surprisingly common (73). In light of this information the DNA sequence directly preceding the plastocyanin gene in pBR322 was analyzed for a possible promoter sequence. In the search for a possible promoter three elements were considered to be essential.

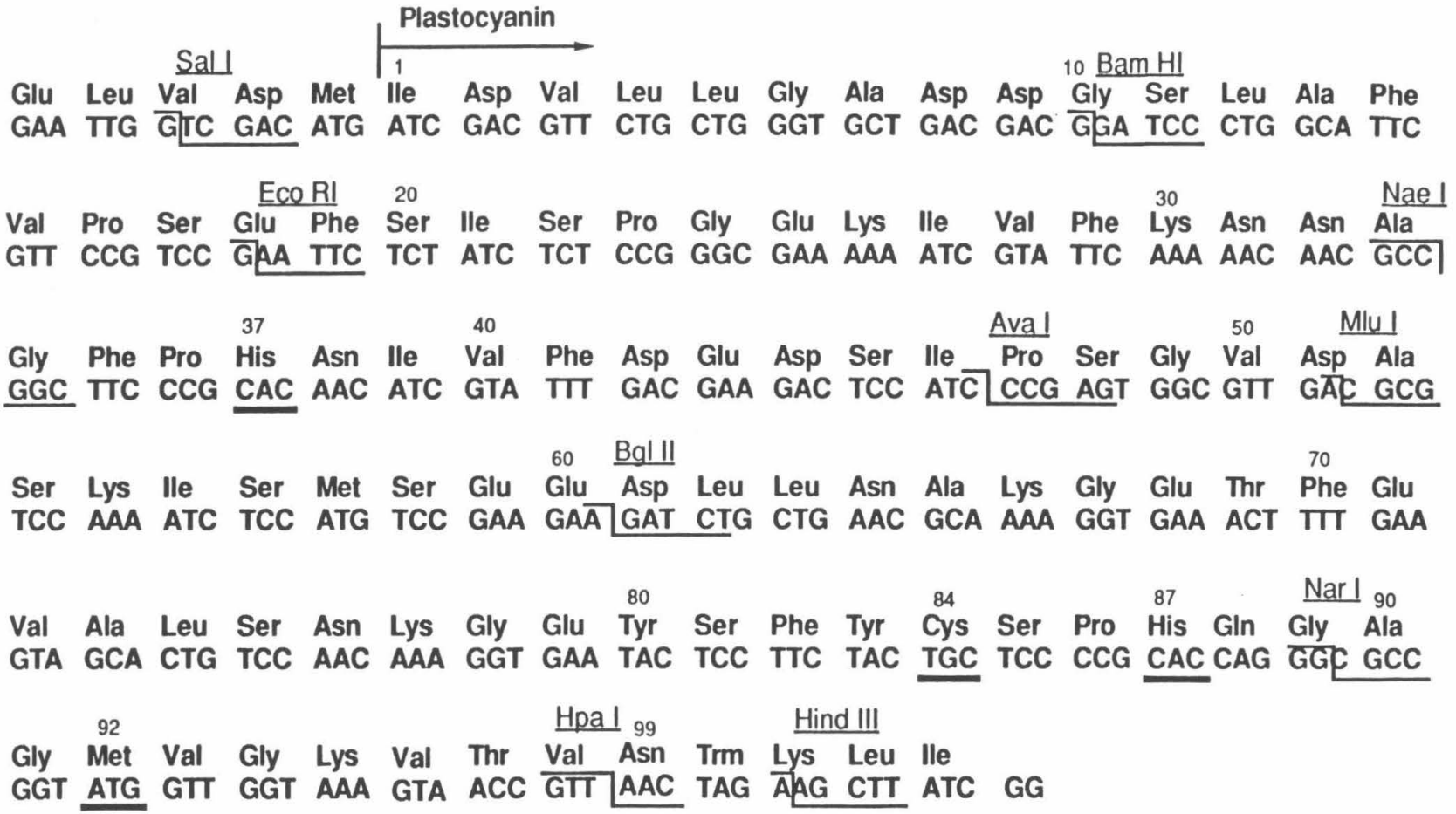
(1) Shine-Dalgarno sequence (ribosome binding site).

(2) -10 sequence for binding of RNA polymerase (Pribnow box).

(3) -35 sequence for binding of RNA polymerase.

Figure 25

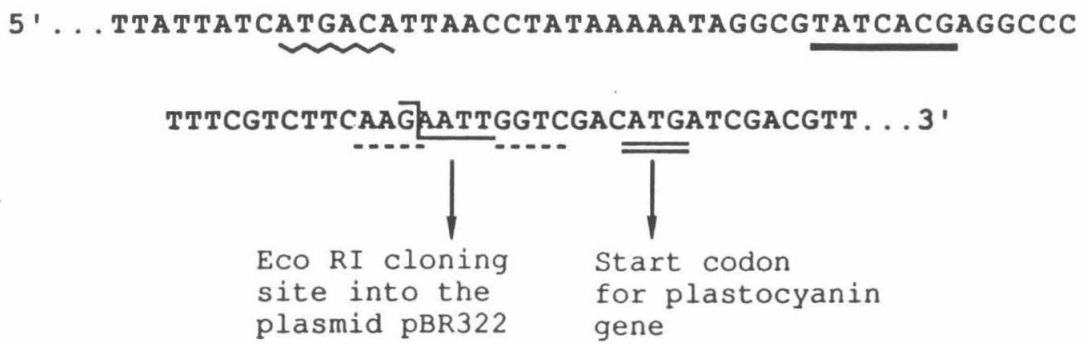
DNA sequence and translation of the completed gene for poplar leaf plastocyanin in pLasto 2. All of the unique restriction sites are indicated and the four amino acids which bind the copper atom are underlined.


Plastocyanin
Sal I
Bam HI
Eco RI
Nae I
Ava I
Mlu I
Bgl II
Nar I
Hpa I
Hind III
Glu Leu Val Asp Met Ile Asp Val Leu Leu Gly Ala Asp Asp Gly Ser Leu Ala Phe
GAA TTG GTC GAC ATG ATC GAC GTT CTG CTG GGT GCT GAC GAC GGA TCC CTG GCA TTC
Val Pro Ser Glu Phe Ser Ile Ser Pro Gly Glu Lys Ile Val Phe Lys Asn Asn Ala
GTT CCG TCC GAA TTC TCT ATC TCT CCG GGC GAA AAA ATC GTA TTC AAA AAC AAC GCC
Gly Phe Pro His Asn Ile Val Phe Asp Glu Asp Ser Ile Pro Ser Gly Val Asp Ala
GGC TTC CCG CAC AAC ATC GTA TTT GAC GAA GAC TCC ATC CCG AGT GGC GTT GAC GCG
Ser Lys Ile Ser Met Ser Glu Glu Asp Leu Leu Asn Ala Lys Gly Glu Thr Phe Glu
TCC AAA ATC TCC ATG TCC GAA GAA GAT CTG CTG AAC GCA AAA GGT GAA ACT TTT GAA
Val Ala Leu Ser Asn Lys Gly Glu Tyr Ser Phe Tyr Cys Ser Pro His Gln Gly Ala
GTA GCA CTG TCC AAC AAA GGT GAA TAC TCC TTC TAC TGC TCC CCG CAC CAG GGC GCC
Gly Met Val Gly Lys Val Thr Val Asn Trm Lys Leu Ile
GGT ATG GTT GGT AAA GTA ACC GTT AAC TAG AAG CTT ATC GG
1
10
20
30
37
40
50
60
70
80
84
87
90
92
99

The analysis for a possible promoter sequence is outlined in Figures 26 and 27. As a result a good match was made between the sequence in the plastocyanin-pBR322 construction and the consensus sequences including the spacing between promoter elements (74). If the plastocyanin was in fact lethal to *E. coli* then selection for mutant proteins could explain the high frequency of mutants observed. If one assumes this to be the case, a mutation in the promoter sequence could allow an *E. coli* transformed with the correct sequence for plastocyanin on a plasmid to grow since the plastocyanin would not be expressed or expressed at very much lower levels. To test this theory, plasmids pLasto 1 and pLasto 2 were sequenced in the region believed to contain the promoter sequence. Recall pLasto 1 contains an addition of a T which creates a termination codon at a critical position in the protein sequence; pLasto 2 has the correct sequence for the entire plastocyanin gene.

DNA Sequencing

The sequencing strategy was analogous to that which has been discussed previously. Fifteen μg of each plasmid was digested with Eco RI, the linear band was visualized by ethidium bromide fluorescence, excised, electroeluted and ethanol precipitated. The pellet was redissolved in 0.25 mM Tris, pH 7.5, and the sample divided in half. One half of the isolated DNA was treated with calf alkaline phosphatase followed by phenol extraction and ethanol precipitation. The pellet was redissolved in buffer appropriate for a kinase reaction which was initiated by the addition of T4 polynucleotide kinase and γ^{32}P-ATP. The other half of the sample was labelled in a fill in reaction initiated with Klenow, α^{32}P-ATP and non-radioactive TTP in order to complete the strand. Both labelled fragments were ethanol precipitated and digested with the restriction enzyme Pvu I. The reaction mixture was loaded onto a 1.2% agarose gel, the 626 base pair band was visualized, excised,



------ Possible Shine-Dalgarno sequence

——— Possible -10 sequence

~~~ Possible -35 sequence

Consensus sequence of promoter elements

Figure 26
~~~

Shine-Dalgarno Sequence		Distance from start codon
5'...TAAGGAGGTGATC...3'	Consensus Sequence	4 - 10 bases
5'...TAAAAAGGGTATC...3'	trp Promoter	9 bases
5'...ACAGGAAACAGAA...3'	lac UV5 Promoter	4 bases
5'...CAAGAATTGGTCG...3'	Proposed Promoter	4 bases

-10 Sequence		
5'...TATA$^{A}_{T}$A$^{A}_{T}$...3'	Consensus Sequence	
5'...TTAACTA...3'	trp Promoter	
5'...TATAATG...3'	lac UV5 Promoter	
5'...TATCACG...3'	Proposed Promoter	

-35 Sequence		Distance from -10 sequence
5'...TTGACA...3'	Consensus Sequence	16 - 19 bases
5'...TTGACA...3'	trp Promoter	17 bases
5'...TTTACA...3'	lac UV5 Promoter	18 bases
5'...ATGACA...3'	Proposed Promoter	20 bases

Sequence which precedes the plastocyanin gene as it was constructed in pBR322

Figure 27

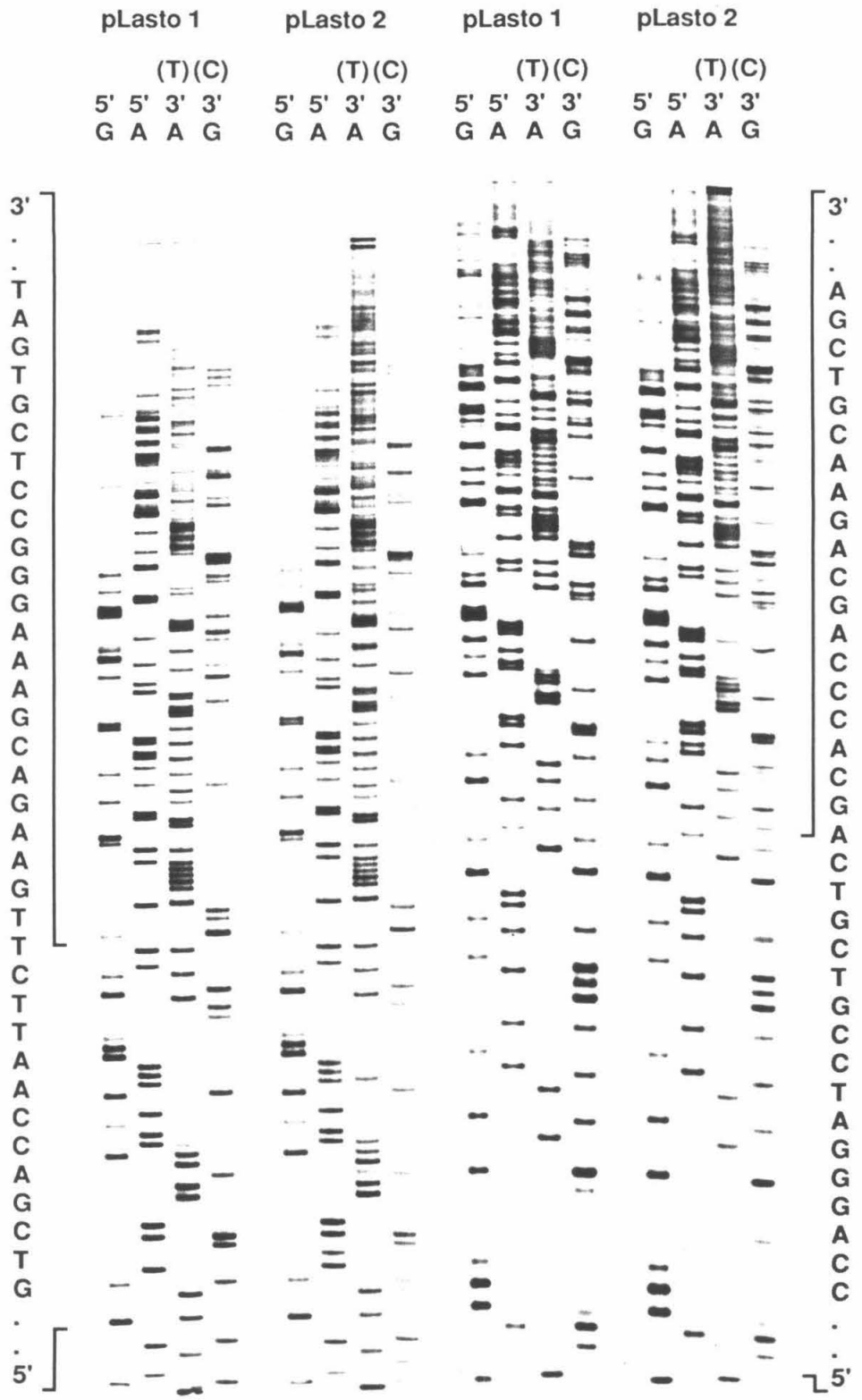
electroeluted and ethanol precipitated. The amount of radioactivity in each pellet was approximated and the G and A specific reactions were performed on each of the 5' and 3' labelled fragments. Following treatment with piperidine the reactions were dissolved in formamide loading buffer and uniform amounts of radioactivity loaded onto an 8% denaturing polyacrylamide gel, autoradiographed and the results analyzed as shown in Figure 28.

Results

No mutations were observed in the proposed promoter sequence of either plasmid. This can be explained in a number of ways, some of the most obvious include: (1) there is no sequence in the plasmid which is recognized as a promoter by the bacterium and the mutations found in the structural gene for plastocyanin were random events; (2) there are other factors, such as a mutation farther upstream in pLasto 2, which reduces the expression of plastocyanin from this plasmid and thereby reduces its adverse effects on the growth of *E. coli*; (3) there is another sequence which is recognized as a promoter in these plasmids which does contain a down mutation in pLasto 2. Sequencing of both plasmids more completely in this region should confirm or eliminate some of these possibilities.

Figure 28

Sequence analysis of the DNA which precedes the start codon for the plastocyanin gene in pLasto 1 and pLasto 2. Autoradiogram of a standard 8% polyacrylamide sequencing gel. The DNA fragments were isolated and radioactively labelled with ^{32}P as described in the experimental section. Cleavage reactions specific for guanine (G) and adenine (A) were used in order to determine the sequence. The DNA sequences in this region of the plasmids were identical.


pLasto 1
pLasto 2
pLasto 1
pLasto 2
(T) (C)
5' 5' 3' 3'
G A A G
3'
. .
T A G T G C T C C G G G A A A G C A G A A G T T C T T A A C C A G C T G
. .
5'
A G C T G C A A G A C G A C C C C A C G A C T G C T G C C T A G G G A C C
. .
5'

Conclusion

The strategy developed during this project for the construction of a gene for poplar leaf plastocyanin has proven very successful. After this first successful gene synthesis in these laboratories, two more genes, α-lytic protease and azurin, were constructed using the same strategy. The general method of stepwise construction should allow for the synthesis of very long genes with great fidelity due to the opportunity to screen the plasmid with each new DNA insert. The same restriction sites designed into the gene sequence, are used for construction and later cassette mutagenesis of the completed gene. The ability to perform cassette mutagenesis will greatly facilitate structure/function studies of plastocyanin in order to elucidate some of the unique redox and spectral properties of the blue copper proteins.

Thorough sequencing of the completed gene revealed a mutation in the original construct, as well as a second mutation following cassette mutagenesis. This high frequency of mutants and the relatively low number of transformants observed following the last step of gene construction, may indicate that *E. coli* has difficulty tolerating the plastocyanin gene product. If this is indeed the case the system used to express this plastocyanin gene must be chosen carefully as discussed in Chapter 2.

Experimental Procedures

Ultraviolet-vislble (UV-Vis) spectra were recorded on a Beckman Model DU7 Spectrophotometer.

High pressure liquid chromatography (HPLC) was performed with a Waters system, model 6000A pumps, model 440 absorbance detector, model 660 solvent programmer, and model U6K injector. Reverse phase HPLC was performed on Waters μ-bondpack C_{18} reverse phase HPLC column.

Lyophilizations were performed on a Labconco lyophilizer or a Savant Speedvac Concentrator. 8% Polyacrylamide gels were dried on a Bio-Rad model 483 slab dryer and autoradiography performed with Kodak X-Omat AR film. Photographs of agarose gels were taken with Polaroid 667 film.

Polyacrylamide gels were run in 1 X TBE electrophoresis buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA). Lyophilized samples were redissolved in 1 X Formamide loading buffer (80% v:v formamide in water, 50 mM Tris-borate, pH 8.3, 1 mM EDTA, 0.1% w:v bromophenol blue) before loading onto denaturing polyacrylamide gels. Samples to be loaded onto non-denaturing polyacrylamide gels were dissolved in 1/10 volume of 10 X glycerol loading buffer (30% v:v glycerol in water, 0.1% w:v bromophenol blue). Agarose gels were run in 1 X TAE electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA). Samples were loaded in 1/5 volume 5 X agarose loading buffer (50% v:v glycerol in water, 5 mM NaOAc, 1 mM EDTA, 0.05% w:v bromophenol blue). In order to visualize the DNA bands on agarose gels 5 μl of 10 mg/ml ethidium bromide was added to each 100 ml of gel volume while the gel was being prepared. Agarose for preparative DNA gels was purchased from International Biotechnologies, Inc.

Computer modelling studies were carried out on an Evans and Sutherland PS340E/μVaxII system using Biograf version 1.32 software.

Geometry optimization was performed using the Biograf Dreiding default parameters and an energy minimization program. All chemicals were the best available grade and used without further purification unless otherwise stated. Water was pretreated with an organic removal cartridge (Corning) and doubly distilled. For all reactions involving DNA and/or *E. coli*, all buffers, growth media glassware and pipette tips (Rainin) were autoclaved prior to use.

Manual Synthesis of Oligonucleotides

All solvents (reagent grade) are commercially available. When anhydrous solvents were required, reagent grade solvents were kept in the presence of a molecular sieve (Aldridge) for one week before use. Nucleotide monomers were purchased from American Bio Nuclear and silica gel was purchased from Vydac. Derivatized silica gel, deoxyribose nucleoside phosphoramidites and most solutions were previously prepared in our laboratory according to literature methods (64-66).

(1) DMT Removal. To remove the dimethoxytrityl group a 5% dichloroacetic acid/toluene solution was used. This was immediately followed by 1 ml of 5% diisopropylethylamine in toluene in order to neutralize any remaining acid.

(2) Coupling Reaction. For the coupling reaction, tetrazole, a very weak acid is added to activate the phosphoramidite. 1.05 grams of tetrazole was dissolved in 30 ml of acetonitrile. 40 μmoles of the nucleoside phosphoramidite was dissolved in 0.4 mls of the tetrazole solution and shaken for 5 minutes (first three couplings shaken for 10 minutes).

(3) Capping. The capping reaction is necessary to cap any unreacted 5′-hydroxyl groups. 0.1 ml of acetic anhydride was dissolved in 1 ml of a solution of dimethlyaminopyridine in tetrahydrofuran:2,6-lutidine (3.5 grams DMAP,

20 ml lutidine, 80 ml THF). This solution was added to the reaction and shaken for 2 minutes.

(4) Oxidation. A solution of 0.2 M iodine in THF:2,6-lutidine:water 1:2:1:1 was prepared. 1.5 ml was added to the reaction and shaken for 3 minutes.

Cycle Schedule

(1)	Toluene wash	2 x 30 sec	1.5 ml
(2)	DCA	2 x 45 sec	1.5 ml
(3)	DIPEA	1 x 30 sec	1.0 ml
(4)	CH_3CN wash	5 x 30 sec	1.5 ml
(5)	Coupling	1 x 5 min	0.4 ml
(6)	CH_3CN wash	2 x 30 sec	1.5 ml
(7)	Capping reaction	1 x 2 min	1.1 ml
(8)	CH_3CN wash	2 x 30 sec	1.5 ml
(9)	Oxidation	1 x 2 min	1.5 ml
(10)	Methanol wash	5 x 30 sec	1.5 ml

Yields. The coupling efficiency at each cycle was measured by the absorbance of the trityl cation at 498 nm. The overall yields for the two oligonucleotides synthesized manually were determined. 1P, a 29 mer had an overall yield of 24% which is approximately 95% per cycle. 2P, also a 29 mer had an overall yield of 29.4% which is approximately 96.8% per cycle.

Deprotection. Silica gel with 0.25 μmoles of completed sequence was shaken for 1 hour in 5 ml of a solution of thiophenol:triethylamine:dioxane (1:2:2). Short vortexing was used to complete mixing. The mixture was centrifuged in a clinical centrifuge and the supernatant was removed. The silica gel was then washed with 5 mls of 1 X dioxane; 5 X methanol; 1 X ether and air dried in a hood for one half hour. 1.5 ml of a saturated ammonium hydroxide solution was added and the mixture shaken for 4 hours. The mixture was

centrifuged and the supernatant transferred to a clean tube. The silica gel was then washed with an additional 1 ml of saturated ammonium hydroxide, centrifuged and this supernatant added to the first. The combined supernatant was incubated at 50°C overnight (15 hours). Ammonium hydroxide was removed by evaporation *in vacuo*. Seventeen drops (from a Pasteur pipette) of 80% acetic acid was added and allowed to react for 15-20 minutes vortexing occasionally. The acetic acid was removed *in vacuo* and the oligonucleotide redissolved in 0.55 ml of 0.1 M triethylammoniumacetate (TEAAc). This was separated into 5 tubes (0.05 μmoles/tube) and lyophilized.

Automated Oligonucleotide Synthesis

All subsequent oligonucleotides discussed in this thesis were synthesized on an Applied Biosystems 380A automatic DNA Synthesizer. This machine uses the same phosphoramidite chemistry as previously described with a few modifications. The first wash is nitromethane in place of toluene and the cleavage is done in 3% DCA/CH_3NO_2 in place of DCA/toluene. Reactions were carried out on a 1 μM scale and the machine employs an automatic deprotection cycle which was used unless otherwise stated. The oligonucleotides were synthesized using the standard reaction program; phosphoramidite bases and reagents were supplied by the manufacturer. The synthesis was monitored by the amount of dimethoxytrityl group released after each cycle.

HPLC Purification of Oligonucleotides

Oligonucleotides 1P, 2P and 3P were purified using a Waters HPLC system and μ-bondpack C_{18} reverse phase HPLC column. Conditions were optimized for separation in each case using a linear gradient of acetonitrile in 0.1 M TEAAc.

1P 12-13% over 1hours.

2P 12-13% over 1 hour

3P 11-13% over 1 hour, 15 minutes

0.05-07 μmoles were loaded onto the column and the appropriate peak collected. The solvent was evaporated *in vacuo* to approximately 100 μl, resuspended in 200 μl of water and lyophilized to dryness. These sequences were first purified while still containing the trityl groups on the 5′ ends to separate them from sequences that were capped during synthesis. The trityl groups were removed after this initial purification and the oligonucleotides were repurified by HPLC.

Gel Purification of Oligonucleotides

3P, 4P and all subsequent oligonucleotides were purified using preparative polyacrylamide gel electrophoresis. A preparative gel of 15% polyacrylamide (1:20 crosslinked) 42% urea was prepared. The gel was 2 mm x 20 cm x 38 cm with wells 2 cm long. Approximately 0.025 μmoles of each oligonucleotide were loaded per well. The gel was run at 500 V for 10-15 hours. The bands were visualized with a fluorescent silica gel plate beneath the gel and illuminating with a hand held UV light (short wave). The desired band was excised from the gel. The gel slice was crushed, placed in a minimum amount of 0.2 M NaCl and eluted overnight at either room temperature or 50°C. This mixture was then filtered and the liquid collected. Oligonucleotides 3P, 4P and 1-12 were passed through a Sephadex G-25-150 column to remove any remaining polyacrylamide and perform a buffer exchange. The Sephadex was swollen in 0.1 mM Tris, 0.01 mM EDTA and the columns were poured in 5 ml plastic syringes with a glass wool plug and spun in a clinical centrifuge. 1 ml of sample was loaded per column, spun and the liquid collected. The columns were chased with 250 μl of column buffer; the

fractions for each oligonucleotide were collected and lyophilized to dryness. All other purified oligonucleotides were dialyzed at 4°C in Spectrapore 7, 7.6 mm diameter 2000 molecular weight cutoff (MWCO) dialysis tubing (Spectrum Medical Industries) against 3 X 3500 ml of 0.25 mM Tris-HCl, pH 7.5. The concentration of purified oligonucleotides were determined by their UV absorbance at 260 nm in a 1 cm path length cell by the relationship 1 O.D. at 260 nm corresponds to approximately 20 μg/ml for oligonucleotides. The ratio of the O.D. readings at 260 nm and 280 nm ($O.D._{260}/O.D._{280}$) gives an estimate for the purity of the DNA (46). A pure preparation of DNA has an $O.D._{260}/O.D._{280}$ of 1.8 and and oligonucleotides were not used unless this ratio of ≥ 1.8.

Polymerization Strategy for Plastocyanin Gene Construction

5′ End-Labelling

Unpurified 1P, 2P, 3P and 4P, HPLC purified 1P, 2P and 3P and gel purified 3P and 4P were individually phosphorylated at their 5′ termini. 50 pmoles of oligonucleotide was placed in 7 μl water and 1 μl 10 X kinase buffer (0.7 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$). 1 μl of γ^{32}P-adenosine 5′-triphosphate (γ^{32}P-ATP) (>7,000 Ci/mmole, New England Nuclear) and 1 μl of T4 polynucleotide kinase (10 units/μl, New England Biolabs). The reactions were incubated for 30 minutes at 37°C, followed by the addition of 1 μl (10% volume) of 3M sodium acetate and 30 μl (3 X volume) ethanol. The reaction was chilled on dry ice for 5 minutes and then spun in an Eppendorf microcentrifuge (Model 5412, Brinkman) at 10,000 revolutions per minute for 10 minutes at 4°C. The supernatant was removed and the radioactive pellet was dried briefly *in vacuo*.

Annealing

The purified oligonucleotide radioactive pellets were dissolved in 12 µl water and 2 µl 10 X Klenow buffer (60 mM Tris-HCl, pH 7.4, 500 mM NaCl, 50 mM $MgCl_2$). 50 pmoles of complementary purified oligonucleotides were placed in a final volume of 12 µl water, 2 µl 10 X Klenow buffer, added to their radioactively labelled partner and placed in a 90°C water bath for 3 minutes then removed from the heat. The oligonucleotides were allowed to remain in the water bath until the temperature came to room temperature.

Polymerization Reactions for Second Strand Synthesis

To each tube of annealed oligonucleotide pairs was added 4 µl of dNTPs (a mixture of 1 mg/ml each), 3 µl of 50 mM DTT and 3 µl of the Klenow fragment of DNA polymerase I (1 unit/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at room temperature for 30 minutes and was stopped by the addition of 2 volumes of phenol. The mixture was mixed to an emulsion, centrifuged for 15 seconds in an Eppendorf microcentrifuge and the upper aqueous phase transferred to a clean tube. This was repeated with 2 volumes of chloroform:isoamyl alcohol (24:1). Finally, 3 volumes of ether was used to extract any traces of phenol, to the lower aqueous phase was added 10% volume of 3 M sodium acetate, and 3 volumes of ethanol. The mixture was chilled on dry ice for 5 minutes and then spun in a microcentrifuge for 10 minutes, and the supernatant was removed and the pellet was dried briefly *in vacuo*.

All samples were dissolved in glycerol loading buffer and loaded onto a 20 cm 15% non-denturing polyacrylamide gel for analysis.

Exonuclease Reactions

To the pellets from the polymerase reactions was added 30 µl water, 4 µl 10 X Klenow buffer, 3 µl 50 mM DTT and 1 µl of the appropriate dNTP

(10 mM) as indicated in Design of System. The reaction was initiated by the addition of 2 µl of Klenow (4 units/µl, Bethesda, Research Labs). The reaction was allowed to proceed at 4°C overnight. The mixture was then loaded onto a 12.5% denaturing polyacrylamide gel (38 cm X .2 mm). The gel was run at 500 V for 10 hours and autoradiographed. No difference in length was apparent in the oligonucleotides reacted with exonuclease when compared with the original oligonucleotide standards loaded onto the same gel. Reaction conditions were varied in concentration of enzyme, 2 units/reaction to 50 units/reaction and temperature, 4°C to 25°C with no apparent exonuclease activity when analyzed by denaturing polyacrylamide gel electrophoresis. The same reaction conditions and analysis were performed using T4 polymerase (3 units/µl, New England Biolabs) and again no exonuclease activity was evident when compared to standards on a denaturing polyacrylamide gel.

Construction of Plastocyanin Gene: Total Synthesis

Oligonucleotide Synthesis

The synthesis and purification of the oligonucleotides have been described previously.

5′ End-labelling of Oligonucleotides

Following UV analysis for purity and concentration oligonucleotides were phosphorylated at their 5′ termini in preparation for ligation. 10 pmoles of each oligonucleotide were placed in 15 µl of water, 2.5 µl of 10 X kinase buffer (0.7 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$), and 3.5 µl of 50 mM DTT. The reaction was initiated by the addition of 2 µl of 10 mM ATP and 2 µl of polynucleotide kinase (12 units/ml, Boehringer Mannheim Biochemicals), the reaction was allowed to proceed for 30 minutes at 37°C.

Annealing of Oligonucleotides

10 pmoles of each kinased oligonucleotide was in 25 µl from the previous reaction. 25 µl of each complementary oligonucleotide was added to its partner, 6 µl of 10 X ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$) and 4 µl of water were also added to the mixture. The mixture was placed in a 90°C water bath, then allowed to cool slowly to room temperature while remaining in the water bath after it was removed from the heat. The concentration of double stranded oligonucleotides was approximately 0.15 pmoles/µl.

Vector Preparation and Ligation

Step 1

Vector Preparation

For the first step, 1 µg of pBR322 (0.35 pmoles (Bethesda Research Labs) was placed in 15 µl of water and 2 µl 10 X medium enzyme buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT). The reaction was initiated by the addition of 1 µl Ava I (4 units/µl, Bethesda Research Labs). After incubation at 37°C for 1 hour, 1 µl of Eco RI (10 units/µl, Bethesda Research Labs) was added and the reaction was incubated for another hour at 37°C followed by heat inactivation of the enzyme at 65°C for 10 minutes. This reaction mixture was used in the ligation reaction without further purification.

Ligation

1 µl of the digested pBR322 (0.035 pmoles) and 2.5 µl of each annealed oligonucleotide duplex (0.4 pmoles each) were placed in 28 µl of water, 5 µl 10 X ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$), 3 µl 50 mM DTT and 3 µl 10 mM ATP. The reaction was placed on ice and initiated by the addition of 5 µl of T4 DNA ligase (3 units/µl, Bethesda Research Labs) followed by

incubation at 15°C overnight (15 hours). 10 µl of this reaction was used to transform *E. coli* LS1 without further purification.

Step 2

Vector Preparation

3 µl of 10 X medium enzyme buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT) was added to 25 µl of pStep 1 (plasmid DNA isolated after transformation to be discussed later). The reaction was initiated by the addition of 1.5 µl of Eco RI (20 units/µl, New England Biolabs) and incubated at 37°C for one hour, then 1.5 µl of Hind III (20 units/µl, New England Biolabs) was added and the reaction incubated for another hour at 37°C, followed by heat inactivation of the enzyme at 65°C for 10 minutes. This digested vector was used in the ligation without purification.

Ligation

20 µl of digested plasmid was mixed with 2.5 µl of each annealed oligonucleotide duplex (0.4 pmole each), 5 µl of 10 X ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$), 3 µl 10 mM ATP and 12 µl water. The reaction mixture was placed on ice, initiated by the addition of 5 µl of T4 DNA ligase (3 units/µl, Bethesda Research Labs) and incubated overnight at 15°C. The mixture was then phenol extracted with 2 volumes of phenol and the upper aqueous phase transferred to a clean tube; this was followed by two consecutive ether extractions with 3 volumes of ether. Sodium acetate was added to a concentration of 0.3 M, followed by the addition of 3 volumes of ethanol. The mixture was chilled on dry ice for 15 minutes, followed by spinning in a microcentrifuge for 10 minutes at 10,000 rpm. The supernatant was removed and the pellet dried briefly. The pellet was redissolved in 20 µl 0.25 mM Tris-HCl, pH 7.5, and 10 µl of this was used to transform *E. coli* LS1 cells.

Step 3

Vector Preparation

3 µl of 10 X medium enzyme buffer (0.1 M Tris-HCl, pH, 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT) was added to 25 µl of pStep 2 (plasmid DNA isolated after transformation will be discussed later). The reaction was initiated by the addition of 2.0 µl Ava I (4 units/µl New England Biolabs) and incubation at 37°C for one hour; at this time 1.0 µl of Hind III (20 units/µl, New England Biolabs) was added and the reaction was allowed to proceed at 37°C for another hour, followed by heat inactivation at 65°C for 1 hour. This reaction mixture was used in the ligation reaction without purification.

Ligation

20 µl of the digested plasmid was mixed with 2.5 µl of each annealed oligonucleotide duplex (0.4 pmole each), 5 µl of 10 X ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$), 3 µl 10 mM ATP and 12 µl water. The mixture was placed on ice and the reaction initiated by the addition of 5 µl of T4 DNA ligase (3 units/µl, Bethesda Research Labs) followed by incubation at 15°C overnight. The mixture was then extracted once with 2 volumes of phenol, twice with 3 volumes of ether followed by an ethanol precipitation from 0.3 M sodium acetate. The pellet was redissolved in 20 µl 0.25 mM Tris-HCl, pH 7.5 and 10 µl of this DNA was used to transform *E. coli* LS1.

Step 4

Vector Preparation

7 µl of 10 X Nar I buffer (60 mM Tris-HCl, pH 7.4, 60 mM $MgCl_2$), 5 µl 50 mM DTT and 24 µl water were added to 30 µl of pStep 3 (plasmid isolated after transformation to be discussed later). The reaction was initiated by the addition of 5 µl Nar I (5 units/µl, New England Biolabs) and incubated at 37° for 10 hours. Due to incomplete digestion it was necessary to separate the

linear DNA from DNA that was nicked or uncut. The reaction was loaded onto a 1.2% agarose gel containing 5 µl/100 mls of gel of 10 mg/ml ethidium bromide. The gel was run for 6 hours at 120 V; a piece of NA-45 DEAE-cellulose (Schleicher and Schuell) was inserted just above, to catch larger DNA, and below the linear band. The gel was run at 150 V for 30 minutes until the linear DNA had run into the lower membrane as judged by ethidium bromide fluorescence. The lower cellulose membrane was placed in a 1.5 ml microcentrifuge tube and just enough high salt NET buffer (1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 8.0) to cover the membrane. The tube was centrifuged for 5 seconds in a microcentrifuge to submerge the whole strip, then incubated at 55°C for 20 minutes with occasional swirling. The buffer was removed and placed in a clean tube; the membrane was washed with an additional 50 µl of buffer (5 minutes at 55°C), which was combined with the first fraction of buffer. The buffer containing the linear DNA was extracted with 3 volumes of water saturated n-butonol to remove residual ethidium bromide followed by ethanol precipitation from 0.3 M sodium acetate to remove excess NaCl. The pellet was redissolved in 40 µl of 0.25 mM Tris-HCl, pH 7.5. To check the purification of the linear band, 5 µl of the purified DNA was loaded onto a 1.2% agarose gel with 5 µl/100 ml gel of 10 mg/ml ethidium bromide next to an unpurified sample. Only linear DNA was apparent in the lane where the DNA band purified with DEAE-cellulose was loaded. 15 µl of the purified linear DNA was combined with 2 µl 10 X medium enzyme buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT). The reaction was initiated by the addition of 2 µl Bgl I (8 units/µl Boehringer Mannheim Biochemicals) and incubated at 37°C for one hour, followed by heat inactivation of the enzyme at 65°C for 10 minutes. This digested vector was used in the ligation reaction without further purification.

Ligation

10 µl of the digested plasmid pStep 3 was combined with 2.5 µl each of the annealed oligonucleotide duplexes (0.4 pmole each), 5 µl 10 X ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$), 3 µl 50 mM DTT, 3 µl 10 mM ATP and 19 µl water. The mixture was placed on ice and the reaction initiated by the addition of 5 µl of T4 DNA ligase (1 unit/µl, Boehringer Mannheim Biochemicals) which was allowed to incubate at 15°C overnight. The reaction mixture was then extracted once with 2 volumes of phenol, twice with 3 volumes of ether and ethanol precipitated from 0.3 M sodium acetate. The pellet was redissolved in 10 µl 0.2 mM Tris-HCl, pH 7.5, all of which was used to transform *E. coli* LS1.

Preparation of Stock Solutions

All phenol used in extractions were purchased from International Biotechnologies, Inc. as a redistilled solid. The solid was melted at 65°C and extracted with 1 M Tris-HCl, pH 8.0, followed by extraction with 0.1 M Tris-HCl, pH 7.8, until the pH of the aqueous phase is 7.6. The phenol is stored under nitrogen in small aliquots and a fresh aliquot is used for each extraction. All ether had been saturated with water prior to use unless otherwise stated. Ampicillin is added from a stock solution which is 10 mg/ml in 0.1 M Tris-HCl, pH 7.8; this stock was kept at -20°C for no longer than 5 days prior to use. Tetracycline stock solution is 15 mg/ml in 50% (v/v) ethanol and water. This solution was stored at -20°C for no longer than a week prior to use.

Transformation

The standard Hanahan transformation was followed as outlined below. 1 ml of L-broth was inoculated with 1 colony of the cell line to be transformed (*E. coli* LS1) and allowed to grow to saturation at 37°C. 250 µl of this

saturated culture was used to inoculate 25 ml of SOB (see transformation stock solutions for all recipes) in a side-arm flask which was pre-warmed to the growth temperature. The flask must be at least 3 times volume. This culture was grown at 37°C to log phase (35-40 Klett $O.D._{600}$). The cells were collected in two 50 ml centrifuge tubes (12.5 ml each) and set on ice for 15 minutes, followed by spinning at 2500 rpm in a Sorvall SS34 rotor for 12 minutes at 4°C. The pellet was resuspended in 1/3 volume (4.2 ml) TFB and mixed very gently followed by spinning at 2500 rpm for 10 minutes at 4°C. The pellet was resuspended in 1/12.5 volume of original volume (1 ml each tube). Fresh DMSO was added to 3.5%, swirled and set on ice for 5 minutes, followed by the addition of 2.25 M DTT to a final concentration of 75 mM. The mixture was swirled and set on ice for 10 minutes, a second aliquot of DMSO was added, swirled and set on ice for 5 minutes. 200 µl samples were placed into large (1.5 ml) Eppendorf tubes which had been chilled on ice. DNA to be transformed was added in a volume of ≤10 µl; the mixture was swirled and incubated on ice for 30 minutes. A heat pulse of 42°C was applied for 90 seconds and the samples were set on ice for 1-2 minutes. This was followed by the addition of 800 µl of SOC (room temperature) and the mixture was incubated at 37°C for 1 hour. The cells were spun for 10 seconds in a microcentrifuge, the pellet was resuspended in 100 µl of SOC and plated onto L-agar plates (100 X 15 mM containing ampicillin at 40 mg/L. The plates were then incubated at 37°C overnight. DNA from the ligation reactions was used in the transformation, as well as ligation mixtures which contained no oligonucleotide duplex inserts in order to approximate the background expected from this DNA. Known dilutions of pBR322 were also transformed in order to check the efficiency of the transformation. LS1 cells which

contained no plasmid DNA were also plated onto the L-agar plates to verify the selection of the antibiotic.

Transformation Stock Solutions

L-agar is L-broth, 1% tryptone (w/v), 0.5% yeast extract (w/v) 10 mM NaCl with 1.5% Bacto-agar (w/v) added. The mixture is made with water and autoclaved. SOB is 2% tryptone (w/v), 0.5% yeast extract, w/v), 10 mM NaCl, 2.5 mM KCl and 10 mM $MgCl_2$, 10 mM $MgSO_4$ in water. The SOB is prepared without Mg^{2+} and autoclaved. A 2 M stock of Mg^{2+} (1 M $MgCl_2 \cdot 6H_2O$ + 1 M $MgSO_4 \cdot 7\ H_2O$) was used to bring the SOB to 20 mM Mg^{2+}. The 2M stock solution was sterile filtered through a 0.22 μm filter prior to use. SOC is SOB with glucose added to a final concentration of 20 mM. The glucose was added from a 2 M stock solution which had been filtered through a 0.22 μM filer. TFB (transforming buffer) is 10 mM K-Mes, pH 6.2, 100 mM $RbCl_2$, 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM $HACoCl_3$. The buffer is made in water, sterilized through a 0.22 μM filter and stored at 4°C. 2.25 M DTT (Boehringer Mannheim Biochemicals) was in 40 mM potassium acetate, pH 6.0, and stored in aliquots at -20°C. DMSO was aliquoted into 200 μl portions and stored at -20°C, and a fresh aliquot was used for each transformation. Drug stock solutions were prepared as previously described.

Plasmid DNA Preparation: Alkaline Lysis

50 ml Culture

Transformed cells are grown to saturation in 50 ml of L-broth. The cells were spun for 5 minutes at 4000 rpm in an SS34 rotor. The supernatant was removed, the pellet resuspended in 2 ml of solution I (see plasmid prep stock solutions) and mixed gently for 5 minutes, followed by the addition of 4 ml solution II. The mixture was swirled gently and placed on ice for 5 minutes, followed by the addition of 3 ml solution III. This was mixed gently but

thoroughly followed by spinning at 13,000 rpm for 10 minutes at 4°C. The supernatant was very carefully removed from the fluffy pellet and 2.5 volumes of ethanol was added to the supernatant at room temperature mixed and allowed to stand at room temperature for 5 minutes. The mixture was spun at 13,000 rpm for 15 minutes at room temperature. The supernatant was removed and the pellet redissolved in 70% (v/v) ethanol (room temperature) followed by spinning at 13,000 rpm for 10 minutes at room temperature. The supernatant was removed and the pellet dried briefly *in vacuo*. The DNA pellet was redissolved in the desired amount of 0.25 mM Tris-HCl, pH 7.5, for further manipulations. The same protocol was followed for smaller DNA plasmid preparations, 1.5 ml (mini-prep). 3 ml of cells are grown overnight in a 15 ml falcon centrifuge tube. The cells were transferred to a 1.5 ml Eppendorf microcentrifuge tube for the plasmid preparation. All spins were performed at 10,000 rpm in a microcentrifuge and volumes of solutions used were as follows: 0.1 ml solution I, 0.2 ml solution II and 0.15 ml solution III. For initial screening of recombinant plasmids 1.5 ml plasmid preparations were used; when a plasmid was shown to contain the correct restriction site map a larger 50 ml preparation of plasmid DNA was performed to obtain enough DNA for the next cloning step. Following the 50 ml plasmid preparation the DNA pellet was dissolved initially in 250 µl of 0.25 mM Tris-HCl, pH 7.5. Aliquots of this plasmid were run on a 1.2% agarose gel with 5 µl/100 ml gel volume of 10 mg/ml ethidium bromide, along with known quantities of plasmid pBR322. An estimate of the concentration of DNA from the plasmid preparation was made based on the amount of ethidium bromide fluorescence when compared to the pBR322 standards. The concentration was then adjusted if necessary to approximately 0.04 pmole/20 µl, for use in the ligation reactions. For step 4 the concentration was approximately 0.1

pmole/20 µl to compensate for any loss of DNA in the purification steps preceding the ligation reaction.

Plasmid Prep Stock Solutions

Solution I is 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0. Lysozyme was added to a concentration of 5 mg/ml just prior to use.

Solution II is 0.2 N NaOH and 1% SDS which were mixed from stock solutions just prior to use. Stock solutions were 1 M NaOH and 10% SDS.

Solution III is 5 M potassium acetate, pH 4.8, which was added ice cold. This solution was prepared by combining 60 ml of 5 M potassium acetate with 1.5 ml of glacial acetic acid and 28.5 ml water. This creates a solution which is 3 M with respect to potassium and 5 M with respect to acetate.

Restriction Digest Analyses

pStep 1

For analysis of the DNA following Step 1, 1.5 ml plasmid preparations were done on 10 of the colonies obtained after transformation of *E. coli* with the ligation mixture. The DNA pellet was redissolved in 60 µl 0.25 mM Tris-HCl, pH 7.5. 5 µl of this DNA was combined with 1 µl 10 X medium enzyme buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT) for Eco RI, Hind III, Bam HI, Ava I or 1 µl 10 X high enzyme buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 1 M NaCl, 10 mM DTT) for Sal I followed by 3 µl water and 1 µl enzyme in either case. The enzymes used to analyze pStep 1 were Ava I (4 units/µl, New England Biolabs), Hind III (20 units/µl, New England Biolabs), Eco RI (10 units/µl, Bethesda Research Laboratories), Bam HI (8 units/µl, Boehringer Mannheim Biochemicals) and Sal I (10 units/µl, Boehringer Mannheim Biochemicals). The reactions were incubated at 37°C for 1 hour. Undigested plasmid DNA was also loaded onto the gel as a standard. All of the reactions were simultaneously performed on 0.5 µg of

pBR322 (0.5 µg/µl, Bethesda Research Laboratories) in a 10 µl reaction. 5 µl of the reaction mixtures were loaded onto a 1.2% agarose gel with ethidium bromide and run for 3 hours at 100 V. Seven of the ten plasmids showed the correct restriction digest pattern. One of these plasmids was used for the next cloning step (pStep 1).

pStep 2

In pStep 2 the two oligonucleotide duplexes added an Ava I site and a Nae I site to the plasmid. 1.5 ml plasmid preparations were performed after transformation of the ligation mixture into *E. coli* LS1. The pellet was redissolved in 60 µl 0.25 mM Tris-HCl pH 7.5. 5 µl of this DNA was used in 10 µl restriction digest reactions with 1 µl 10 X medium enzyme buffer and 1 µl of enzyme. The enzymes used to analyze pStep 2 were Nae I (5 units/µl, New England Biolabs), Ava I (4 units/µl, New England Biolabs) and Eco RI (20 units/µl, New England Biolabs). Standards of pBR322 (0.5 µg) were digested in simultaneous 10 µl reactions. 5 µl of the mini-prep reactions, 1 µl of the pBR322 reactions and undigested plasmid standards were loaded onto a 1.2% agarose gel with ethidium bromide. The gel was run at 100 V for 3 hours.

pStep 3

The two oligonucleotide duplexes inserted in Step 3 added a Nar I site, Mlu I site, Hpa I site, and a Bgl II site to the plasmid. Following a 1.5 ml plasmid preparation from the cells transformed with the ligation mixture, the pellet was redissolved in 60 µl of 0.25 mM Tris-HCl, pH 7.5. 5 µl of this DNA was used for restriction digest analysis, with 1 µl of 10 X medium enzyme buffer (Mlu I, Hind III, Hpa I, Bgl II) or 1 µl of 10 X Nar I buffer (60 mM Tris-HCl, pH 7.4, 60 mM $MgCl_2$, 10 mM DTT) and 1 µl of enzyme. The enzymes used were Nar I (4 units/µl, New England Biolabs), Mlu I (8 units/µl, New

England Biolabs), Bgl II (8 units/μl, Boehringer Mannheim Biochemicals) and Hind III (20 units/μl, Boehringer Mannheim Biochemicals). The reactions were incubated at 37°C for 1 hour. 5 μl of each reaction mixture was loaded onto a 1.2% agarose gel with ethidium bromide along with 1 μl samples of pBR322 digested in analogous reactions and undigested standards of each plasmid. The gel was run at 100 V for 3 hours.

pStep 4

The two oligonucleotide duplexes inserted at this step did not add any new restriction sites to the plasmid so analysis of the plasmid for the DNA insert was based on a difference in size between plasmid pStep 3 and pStep 4. Following a 1.5 ml plasmid preparation from *E. coli* LS1 cells transformed with the ligation mixture, the pellet was redissolved in 60 μl of 0.25 mM Tris-HCl, pH 7.5. 5 μl of this DNA was combined with 2 μl of 10 X high salt buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 1 M NaCl, 10 mM DTT) for the Pst I, Hind III digest or 2 μl 10 X Nar I buffer (60 mM Tris-HCl, pH 7.4, 60 mM $MgCl_2$, 10 mM DTT) for the Nar I digest, 10 μl water and 2 μl total enzyme were added in either case (1 μl of each Pst I, incubated at 37°C for 1 hour followed by 1 μl of Hind III incubated for another hour at 37°C or 2 μl of Nar I incubated for 2 hours at 37°C). 5 μl of these 10 μl reaction mixtures were loaded onto a 1.2% agarose gel with ethidium bromide and run at 100 V for 3 hours.

<u>CsCl Purification of Plasmid DNA</u>

5 ml of L-broth containing 50 mg/L of ampicillin was inoculated with a single colony from the transformation in step 4 and was incubated overnight at 37°C. This culture was used to inoculate 500 ml of M9CA + uridine + thiamine + ampicillin (see CsCl stock solutions) which was grown to mid-log phase. Chloramphenicol was added to a final concentration of 170 mg/liter

and incubated with shaking for 12 to 18 hours at 37°C. The cells were harvested in two 250 ml centrifuge bottles by spinning for 5 minutes at 6,000 rpm in a GSA rotor, the supernatant removed and the cells were gently resuspended in 40 ml of 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, followed by centrifugation for 5 minutes at 6,000 rpm. This was followed by a standard plasmid preparation as described previously. The volumes of solutions for each 250 ml of cells were 10 ml solution I, 20 ml solution II and 15 ml solution III. All spins were performed in a GSA rotor at the same rpm and times as described for the 50 ml plasmid preparation. The supernatant was removed following the 10 minute spin after the addition of solution III and transferred to a clean tube. No ethanol precipitation was performed on the supernatant which is now referred to as the cleared lysate, but it was used directly in the cesium chloride gradient. 0.95 grams of CsCl and 0.059 ml of 10 mg/ml ethidium bromide was added for each ml of cleared lysate. The mixture was swirled until all CsCl was dissolved and loaded into two Beckman heat sealable VT150 centrifuge tubes which were balanced against each other. The gradient was centrifuged at 45,000 rpm for 20 hours at 17°C in a VTI50 rotor. The tubes were carefully removed from the rotor and the bands were visualized with a long wave UV light. Two bands were visible, the upper band containing nicked plasmid DNA, linear plasmid DNA and *E. coli* DNA, and the lower band which contains supercoiled, closed circular plasmid DNA. The lower band was collected with an 18 gauge needle and syringe punctured through the side of the tube just below the band. The collected DNA mixture was butanol extracted 6 times to remove the ethidium bromide followed by 3 X 3500 ml dialysis against 0.25 mM Tris-HCl, pH 7.5, using Spectropor 7 dialysis tubing 10,000 MWCO (Spectrum Medical Labs). Following dialysis

the purity and concentration of the DNA was estimated by the UV absorption at 260 and 280 nm.

Stock Solutions for CsCl Preparation of Plasmid DNA

10 X M9 salts: 10 g/L NH_4·Cl, 60 g/L Na_2HPO_4, 30 g/L KH_2PO_4, 5 g/L NaCl, the solution was autoclaved and stored at room temperature. For 1 liter of M9 media: 100 ml 10 X M9 salts, 2 ml of 0.5 M $MgSO_4$ (autoclaved separately), 1 ml of 0.1 M $CaCl_2$ (autoclaved separately), 20 ml 20% w/v glucose (filter sterile), 2 ml 1 m/ml thiamine (filter sterile), 10 ml 100 mg/ml uridine (filter sterile), 5 g/liter CASamino acids (Difco), 840 ml water. The CASamino acids and water were mixed, autoclaved and all other ingredients added to the cooled solution as well as ampicillin (50 mg/L). Chloramphenicol is 34 mg/ml in 100% ethanol and ethidium bromide is 10 mg/ml in water.

Sequencing

Maxam-Gilbert Sequencing of pLasto 1

10 µg of CsCl purified pLasto 1 was digested with Hind III by combining 75 µl pLasto 1 (0.15 µg/µl), 10 µl 10 X medium enzyme buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT), 15 µl water and 3 µl Hind III (18 units/µl, Boehringer Mannheim Biochemicals). Another 10 µg of pLasto 1 was digested with Sal I by combining 75 µl pLasto 1, 10 µl 10 X Sal I buffer (0.1 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$, 1.5 M NaCl, 10 mM DTT), 15 µl water, and 4 µl Sal I (12 units/µl Boehringer Mannheim Biochemicals). Both reactions were incubated at 37°C for 2 hours, followed by heat inactivation of the enzyme at 65°C for 10 minutes.

3' End Labelling

To each 100 µl reaction was added 10 µl 50 mM DTT, 2 µl 10 X medium enzyme buffer, 5 µl of radioactive nucleotides (α^{32}P TTP for Sal I digested DNA, α^{32}P dATP for Hind III digested DNA) (3,000 Ci/mmole, Amersham),

and 5 µl Klenow (5 units/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at room temperature for 20 minutes followed by ethanol precipitation from 0.3 M sodium acetate. The pellets were redissolved in 40 µl water and 5 µl 10 X medium enzyme buffer for a second cut with Hind III or 5 µl 10 X Sal I buffer for a second cut with Sal I. To the DNA previously cut with Hind III was added 4 µl Sal I, to the DNA previously digested with Sal I was added 4 µl Hind III, and both reactions were incubated for 3 hours at 37°C.

DNA Isolation

Following the restriction digest reactions the samples were loaded onto a 1.2% agarose gel with ethidium bromide and run at 25 V overnight. The bands were visualized by ethidium bromide fluorescence and the lower band in each lane (~300 bp) was isolated using a DEAE cellulose membrane as previously described. Following extraction with butanol the radioactive samples were dialyzed against 2 x 3500 ml changes of 0.25 mM Tris-HCl, pH 7.5. Maxam-Gilbert sequencing reactions were performed on 6 mR/hr of each labelled DNA fragment according to published methods (68). The reactions were loaded onto an 8% denaturing polyacrylamide gel (0.5 mR/hr per lane) and run for 2.5 hours at 1300 V followed by a second loading of the reactions and another run at 1300 V for 3 hours. The gel was dried and exposed for two days with one intensifying screen.

Sequencing of pLasto 1 and pLasto 2 Using an A Specific Reaction

15 µg of pLasto 1 or pLasto 2 was digested with Sal I or Hind III in 50 µl reactions as described. The plasmid DNA had been purified on a CsCl gradient as described previously and the concentration of pLasto 1 and pLasto 2 was 0.5 µg/µl.

DNA Isolation

The reactions were loaded onto a 1.2% agarose gel with ethidium bromide and run at 100 V for 3 hours. The bands were visualized by ethidium bromide fluorescence under a UV light and the linear band was excised. The DNA was isolated using an Elutrap electro-separation system (Schleicher and Schuell) according to the instructions supplied by the manufacturer, followed by ethanol precipitation from 0.3 M NaOAc. Each pellet was dissolved in 60 µl 0.25 mM Tris-HCl, pH 7.5.

5' End-Labelling

30 µl of isolated linear plasmid DNA was combined with 50 µl water, 10 µl 10 X calf alkaline phosphatase (CAP) buffer (0.5 M Tris, pH 8.0, 10 mM EDTA), 2 µl calf alkaline phosphatase (19 units/µl, Boehringer Mannhein Biochemicals), the reactions were incubated at 37° for 30 minutes followed by the addition of another 2 µl of CAP and incubation at 37° for 30 minutes. The reactions were extracted twice with two volumes of phenol, once with two volumes of chloroform and three times with four volumes of ether, followed by ethanol precipitation from 0.3 M sodium acetate. The pellets were redissolved in 70 µl water, 10 µl 10 X kinase buffer (0.7 M Tris-HCl, pH 7.6, 10 mM $MgCl_2$), 10 µl 50 mM DTT, 3 µl γ^{32}P-ATP (5,000 Ci/mmole, New England Nuclear) and 10 µl polynucleotide kinase (10 units/µl, Boehringer Mannheim Biochemicals). The reactions were incubated at 37°C for 45 minutes followed by ethanol precipitation from 0.3 M sodium acetate.

3' End-Labelling

30 µl of isolated linear plasmid DNA was combined with 5 µl 10 X Klenow buffer (60 mM Tris-HCl, pH 7.4, 60 mM $MgCl_2$, 0.5 M NaCl), 5 µl 50 mM DTT, 15 µl cold (non-radioactive) nucleotides (5 µl each of a 10 mM stock solution final concentration 1 mM each dNTP) (cold dTTP, dCTP, dGTP,

α^{32}P-dATP for Hind III cut DNA and cold dATP, dCTP, dGTP, α^{32}P-TTP for Sal I cut DNA) and 7 µl α^{32}P dNTP (3,000 Ci/mmole, Amersham). The reactions were incubated at room temperature for 20 minutes followed by ethanol precipitation from 0.3 M sodium acetate.

Second Digest

Each labelled fragment was digested with a second enzyme (Hind III cut DNA digested with Sal I, Sal I cut DNA digested with Hind III) in 50 µl reaction as previously described for the Maxam-Gilbert sequencing protocol. The reactions were loaded onto a 1.5% agarose gel with ethidium bromide and run at 100 V for 1.5 hours. The lower band in each lane (300 base pairs) was visualized, excised and the DNA isolated using an Elutrap electro-separation system, followed by ethanol precipitation from 0.3 M sodium acetate.

G Reactions

Approximately 5 mR/hr of labelled DNA fragments were combined with 1 µg sonicated calf thymus DNA, 100 µl 100 mM sodium cocadylate, pH 8.0, and water was added to a final volume of 200 µl. 1 µl of neat dimethylsulfate was added and the reactions incubated for 2 minutes at room temperature. The reactions were stopped by the addition of 50 µl of a stop solution (1.5 M NaOAc, 1.0 M 2-mercaptoethanol and 40 µg/ml sonicated calf thymus DNA) followed by 750 µl ethanol. The solutions were chilled in dry ice for 10 minutes then spun at 12,000 rpm for 10 minutes. The supernatants were removed and the pellets dried briefly *in vacuo*. The pellets were redissolved in 50 µl 10% aqueous piperidine, heated at 90°C for 30 minutes, frozen on dry ice and lyophilized to dryness.

A Reactions

Approximately 5 mR/hr of the ^{32}P labelled DNA fragments were combined with water to a volume of 160 µl, 1 µg of sonicated calf thymus

DNA. 40 µl of a solution containing 10 mM K_2PdCl_4 (Alfa. Corp.) in 100 mM HCl/NaCl, pH 2.0, and the reactions were incubated for 45 minutes at room temperature. The reactions were stopped by the addition of 50 µl of stop solution (see G reaction protocol for recipe), followed by ethanol precipitation and a piperidine work-up as described for the G reaction.

Gel Electrophoresis

All of the lyophilized DNA from both reactions were dissolved in formamide loading buffer so that 2 µl contained approximately 0.5 mR/hr of radioactivity. 2 µl was loaded onto each lane of standard denaturing 8% polyacrylamide sequencing gels. The gels were dried and autoradiography performed in order to analyze the sequence data. A 20% gel was also run using the same reactions to collect sequence data closer to the ^{32}P labelled end of the DNA fragment for pLasto 2.

Sequencing of the pLasto 1 and pLasto 2

DNA Sequence Preceding the Gene for Plastocyanin

15 µg of pLasto 1 and pLasto 2 were digested with Eco RI in the following reactions. 30 µl of plasmid (0.5 µg/µl) was added to 50 µl water, 10 µl 10 X Eco RI buffer (1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl), 5 µl Eco RI (4 units/µl, Boehringer Mannheim Biochemicals) and the reactions were incubated at 37°C for 3 hours. The reactions were loaded onto a 1.2% agarose gel with ethidium bromide and run at 100 V for 3 hours. The linear DNA bands were excised and the DNA isolated in an Elutrap electro-separation system followed by ethanol precipitation. The pellets were redissolved in 60 µl of 0.25 mM Tris-HCl, pH 7.5, and half of the DNA from each plasmid was labelled at the 5′ end with $\gamma^{32}P$-ATP and the other half at the 3′ end with $\alpha^{32}P$-dATP as described previously for pLasto 1 and pLasto 2. Following ethanol precipitation of the labelled DNA the pellets were dissolved in 50 µl

water, 6 µl 10 X high salt enzyme buffer (0.5 mM Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 1 M NaCl, 10 mM DTT) and 5 µl Pvu I (3.5 units/µl, Boehringer Mannheim Biochemicals). The reactions were incubated at 37°C for 3 hours and loaded onto a 1.5% agarose gel which was run at 100 V for 2 hours. The lower bands (~700 base pairs) were excised and the DNA recovered using an elutrap electro-separation system. G and A specific reactions were performed on 10 mR/hr of each DNA fragment and run on a standard 8% polyacrylamide sequencing gel as previously described for pLasto 1 and pLasto 2.

Cassette Mutagenesis

Synthesis, 5' End Labelling and Annealing of Oligonucleotides

Oligonucleotides 13-16 were resynthesized on an ABI automated DNA synthesizer as previously described. The oligonucleotides were purified on a 15% denaturing polyacrylamide gel followed by dialysis against 3 x 3500 ml 0.25 mM Tris-HCl, pH 7.5. The concentration and purity of the DNA was estimated based on UV absorption at 260 and 280 nm. 10 pmoles of each oligonucleotide was kinased at the 5' end in 25 µl reactions using 2 µl of T4 polynucleotide kinase (10 units/µl, New England Biolabs) and annealed to its complementary partner to yield a final concentration of 0.15 pmoles/µl of duplex oligonucleotides as described previously.

Vector Preparation

10 µg of pLasto 1 (10 µl of 1.0 µg/µl CsCl purified DNA) was combined with 10 µl water, 4 µl 10 X Nar I buffer (60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$), 5 µl 50 mM DTT and 4 µl Nar I (4 units/µl, New England Biolabs). The reaction was incubated at 37°C for 5 hours and then loaded onto a 1.2% agarose gel which was run for 3 hours at 100 V. The linear band was excised and the DNA isolated using an elutrap electro-separation system followed by ethanol precipitation. The pellet was redissolved in 25 µl water, 3 µl 10 X

high salt buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 1 M NaCl, 10 mM DTT), 2 µl Bgl II (10 units/µl, Boehringer Mannheim Biochemicals) and incubated for 2 hours at 37°C, followed by ethanol precipitation from 0.3 M sodium acetate. The pellet was redissolved in 80 µl water, 10 µl 10 X calf alkaline phosphatase (CAP) buffer (0.5 M Tris-HCl, pH 8.0, 10 mM EDTA). 2 µl CAP (10 units/µl, Boehringer Mannheim Biochemicals) and the reaction was incubated for 30 minutes at 37°C. The DNA mixture was extracted twice with 2 volumes of phenol, once with 2 volumes of chloroform, three times with 3 volumes of ether followed by ethanol precipitation from 0.3 M sodium acetate. The pellet was redissolved in 500 µl of 0.25 mM Tris-HCl, pH 7.5 and the concentration of DNA was estimated based on the UV absorption at 260 and 280 nm. The mixture was chilled on dry ice and lyophilized to a concentration convenient for further manipulation (0.01 pmoles/µl).

Ligation

3 µl of each annealed oligonucleotide duplex (0.45 pmoles) were combined with 5 µl of the digested plasmid (pLasto 1) (0.05 pmoles), 5 µl water, 2 µl 10 X ligase buffer (0.25 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$, 40 mM β-mercaptoethanol, 4 mM ATP), 2 µl T4 DNA ligase and the reaction was incubated at 15°C overnight. A control reaction was also performed which was the same as described above without any oligonucleotide duplexes added in order to approximate the background expected from any vector religation. 10 µl of the ligation mixtures were transformed into *E. coli* HB101 and plated onto L-agar plates containing ampicillin (50 mg/L). The colonies were grown in L-broth, the plasmid DNA extracted, purified and the sequence analyzed as previously described.

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Chapter 2

Expression of a Synthetic Gene for Plastocyanin in *E. coli*

Introduction

The recent development of *in vitro* recombinant DNA techniques has greatly enhanced the understanding of the structure and function of the genes from many organisms. It has been shown that genes cloned from many different organisms can be expressed in a new host, such as *E. coli*, if the elements which control expression are properly arranged. Unfortunately, any rules which govern the successful expression of foreign genes in *E. coli* are not well understood and as a result experiments must be designed based largely on empirical observations. Since relatively few promoter systems have been used to express foreign genes in *E. coli*, one must learn from trial and error as each promoter is tested systematically for each case. The options are increased as one considers the use of fusion proteins and various protease deficient strains of *E. coli* in order to enhance the stability of the gene product.

Once the protein has been expressed, structure/function studies may be carried out using oligonucleotide directed mutagenesis or cassette mutagenesis in the case of synthetic genes designed specifically for that purpose.

Background

Escherichia coli as a Host for Expression

E. coli has been widely used as the host for expression of foreign genes largely because more is known about the control of gene expression in *E. coli* than in any other organism (1). Expression of a cloned or synthetic gene in *E. coli* requires efficient transcription of the DNA, translation of the resulting mRNA and occasionally post-translational modification of the protein.

Several differences between eukaryotic and prokaryotic DNA must be considered in order for eukaryotic DNA to be successfully expressed in *E. coli*. First of all there are no introns in prokaryotic DNA therefore no splicing enzymes exist in *E. coli*.

Transcriptional signals also differ between eukaryotes and prokaryotes which may not allow recognition by the bacterial RNA polymerase (2, 3). Additional problems include the difference in mRNA structural signals and codon usage between organisms (4). Fortunately, the problems mentioned above may be alleviated by the proper design of a synthetic gene and subsequent cloning into an appropriate expression vector (5).

Transcription

Transcription of synthetic genes can be controlled by cloning the DNA behind a strong (high level of expression) prokaryotic promoter in an expression vector. Several promoters are commonly used for this purpose (1). Three of these promoters were tried in attempts to express the synthetic gene for poplar leaf plastocyanin in *E. coli*. The β-lactamase promoter is a relatively weak, constitutive promoter on the plasmid pBR322 (6, 7), the tac promoter is a hybrid trp-lac promoter that allows stronger, semi-regulated expression (8, 9) and the λP_L and λP_R promoters which provide strong, well regulated expression (10, 11). Several genes derived from genomic DNA and a

few synthetic genes have been expressed using these systems (12-15) as well as the lac promoter (16), the alkaline phosphatase promoter (17) and the bacteriophage T7 RNA polymerase-promoter system (18).

Translation

Translational difficulties including protein modification and stability are more complicated, as the elements which control these processes in *E. coli* are not well characterized. These problems can be overcome to some extent by creating a fusion protein with a prokaryotic gene in the correct reading frame (13, 14, 19). This allows the initiation of translation to be directed by the existing ribosome binding site and initiation codon for maximum recognition of these elements. There is also evidence that short foreign proteins are unstable in *E. coli* and fusion to a larger protein derived from *E. coli* or another prokaryote enhances the stability of these proteins (20).

Eukaryotic proteins may also be stabilized in *E. coli* by including a signal sequence which is cleaved during transport of the processed protein into the periplasmic space, followed by cleavage of the signal sequence to liberate a functional protein. This strategy is somewhat limited because not all proteins can be transported across the membrane (21). Plastocyanin has been cloned as a precursor from white campion (*Silene pratensis*) followed by *in vitro* translation (22, 23). This precursor was shown to be processed in two separate events as it must cross three membranes of the chloroplast in order to arrive at its final site of function. Unfortunately, this signal sequence is very long, 66 amino acids, and it would require a significant effort to add this sequence onto the synthetic gene for plastocyanin, although the possibility was considered.

Other general methods reported to reduce the amount of protein degradation in *E. coli* include the use of *E. coli* strains deficient in the lon gene

product (lon-), which is a major protease responsible for the breakdown of foreign proteins (20, 24) and the joining of cloned or synthetic genes in tandem to create a multiple copy of the protein which can be subsequently cleaved into monomer units (25).

In the case of plastocyanin in particular, two recent articles suggest that the stability of mature plastocyanin in green algae is dependent on the incorporation of copper by the protein. The apoplastocyanin in rapidly degraded in Cu (II) deficient cells. This degradation did not affect the pre-apoplastocyanin but only the mature apoplastoycanin protein (26). This copper dependent degradation has been suggested as a mechanism by which the expression of plastocyanin is regulated at both the level of stable protein and stable mRNA *in vivo* (27).

Synthetic Genes Expressed in *E. coli*

Very few synthetic genes have been successfully expressed in *E. coli* at this time. These include the gene for the hormone somatostatin (19) and the genes for the A and B chains of human insulin (29). These genes were expressed as fusion proteins with the β-galactosidase gene behind the lac promoter. Two synthetic genes expressed as non-fusion proteins are a gene for calmodulin expressed in a commercial vector using the tac promoter (30) and a gene for the human complement fragment C_{5a} expressed using the lac promoter (31).

Design of System

Choice of Expression Vector

Due to the low number of transformants following the final step of the plastocyanin gene construction, and the high frequency of mutants observed during sequence analysis, it was suspected that expression of plastocyanin in *E. coli* may have an adverse effect on the bacterium. With this in mind, several strategies were considered for the expression of the plastocyanin gene in *E. coli*. The first attempt at expression used the relatively weak constitutive, β-lactamase promoter in pBR322 (6, 7). The plastocyanin gene was cloned into pBR322 to create a fusion protein with β-lactamase. When no protein was detected expression vectors which utilize the tac promoter for strong, inducible expression were used in attempts to express the plastocyanin gene. A commercial vector, pKK223-3 (32), contains the tac promoter (8, 9) followed by a multiple cloning site to facilitate the cloning and expression of the protein of interest. The tac promoter is repressed by lac repressor, therefore a cell line which contains the laciq (33) gene is used for expression experiments. The tac promoter can be derepressed by the addition of isopropylthio-β-D-galactoside (IPTG) to the cell culture. The tac promoter, however, is quite "leaky" and produces a significant amount of protein even in the repressed state.

Protein Stabilization

If the plastocyanin does, in fact, have a lethal effect on the bacterial cells it is necessary to use a more tightly controlled expression system. In order to gain more control of the tac promoter a second vector, pHSe6 (34), was used in an attempt to express the plastocyanin gene. This system also utilizes the tac promoter followed by a multiple cloning region, however, the gene for lac repressor (laciq) is also on the plasmid. This allows production of lac repressor

in the same number of copies as the plasmid (28) to increase the element of control over the promoter. When neither of the tac promoter systems resulted in expression of the plastocyanin gene, the possibilities for the lack of expression were analyzed. The most obvious reasons would include extensive proteolysis of the plastocyanin following expression (24-27) and/or a lethal effect of the protein on the *E. coli*. The plastocyanin may act as a scavenger of copper in the bacterial cells and disrupt the balance necessary for proper growth. In order to reduce the chance of proteolysis, the recombinant plasmids were transformed into a cell strain (C600Δlon) which is deficient in a major proteolytic enzyme but does contain the laciq (33) gene to maintain control over the tac promoter. $CuSO_4$ was also added to the growth medium in various concentrations up to 1 mM in order to supplement the amount of copper available to the bacterium. The cells were shown to tolerate concentrations of $CuSO_4$ up to 1 mM with no obvious effect on their growth. Again no expression of plastocyanin was detected by Western blot analysis.

Another strategy was developed to take advantage of systems which utilize a fusion protein construct under the control of a very tightly repressed promoter in order to reduce or eliminate any lethal effect of expressed plastocyanin on the bacterial cells prior to induction. The first vector, pLcII (13, 35), utilizes the λP_L promoter followed by a short region of the lambda cII gene and a multiple cloning site. The second vector, pRIT2T (32) uses the λP_R promoter followed by a relatively long region of protein A and a multiple cloning site. Both promoters remain very tightly repressed in the presence of lambda repressor. A cell line is used to express proteins from these plasmids, which contains a temperature sensitive repressor (C1857). This lambda repressor is fully active at a temperature of 28°C, and can be inactivated by simply raising the growth temperature to 42°C, which allows expression of the

desired protein at a specific growth stage of the bacterium. The shorter fusion protein system (pLcII) produced no detectable plastocyanin, while the longer protein A fusion produced a relatively large quantity of the fusion protein (as judged by Western blot analysis) when the promoter was induced, and no protein was detected in the uninduced state.

Protein Purification

The fusion protein may be purified by the binding of the protein A region to an IgG sepharose column. This is followed by cleavage at an Asp-Pro sequence built into the plasmid or by insertion of the sequence by cassette mutagenesis which encodes the tetrapeptide Ile-Glu-Gly-Arg. This sequence is placed at the junction between the protein A and plastocyanin proteins. This is the cleavage site for the blood coagulation factor X_a which catalyzes the conversion of prothrombin to thrombin (13). As a result the fusion protein is cleaved by factor X_a . The cleaved protein may be purified a second time on an IgG column to separate plastocyanin from the protein A. This system is ideal for the expression and purification of any plastocyanin mutants since it is not dependent on a functional plastocyanin.

Results and Discussion

Expression Vector Constructions

Several attempts were made at expressing the synthetic plastocyanin gene in *E. coli* using different strategies.

Oligonucleotide Preparation

When linkers were necessary to clone the plastocyanin gene into the expression vector, oligonucleotides were synthesized using an ABI automated DNA synthesizer and purified on denaturing polyacrylamide gels. The purified oligonucleotides were phosphorylated at their 5′ termini and annealed with an equimolar concentration of their complementary partner. This produced a duplex structure(s) which contained the appropriate overhang regions to allow ligation of the plastocyanin gene into the expression vector at the desired location.

Vector Preparation

The expression vector and pLasto 2 were digested with the appropriate restriction enzymes for ligation. The restriction digests were loaded onto a 1.2% agarose gel with ethidium bromide for visualization. The correct bands were excised from the gel, electroeluted and ethanol precipitated. The pellets were redissolved in 500 µl of 0.25 mM Tris-HCl, pH 7.5, and the concentration of the DNA estimated by UV absorbance measured at 260 nm (36). The mixture was then chilled on dry ice and lyophilized to a convenient concentration for use in ligation reactions.

Ligation Reactions

Oligonucleotide duplex(es) were combined, in a ten-fold molar excess, with the purified expression vector and plastocyanin gene from pLasto 2. The reactions were initiated by the addition of T4 DNA ligase and reactions were incubated at 15°C overnight. The mixtures were used to transform *E. coli*

cells and plated onto L-agar plates containing the appropriate antibiotic to screen for recombinant plasmids.

Restriction Site Analyses

Following transformation of *E. coli* with the recombinant plasmids, plasmid preparations were performed on antibiotic resistant colonies and the plasmid DNA analyzed. Restriction enzymes were used to confirm the presence of unique sites introduced into the expression vector with the plastocyanin gene and/or screened by a double restriction enzyme digest. This generates fragments of different sizes, that when compared to fragments of the expression vector without the plastocyanin gene, will confirm the position and orientation of the plastocyanin gene in the expression vector. Plasmids which showed the proper pattern following restriction enzyme digests were tested for expression of the plastocyanin protein.

Western Blot Analysis

E. coli containing the recombinant expression vectors were grown in either L-broth or L-broth containing 10-100 μM $CuSO_4$ and the particular promoter was induced. Cells were harvested and resuspended in protein sample buffer and heated to 95°C for 5-30 minutes to lyse the cells. The lysed cell mixture was loaded onto a standard 12-15% polyacrylamide stacking gel containing SDS, and run at constant current for 6-18 hours. The proteins were then transferred to a piece of DEAE nitrocellulose in preparation for antibody screening.

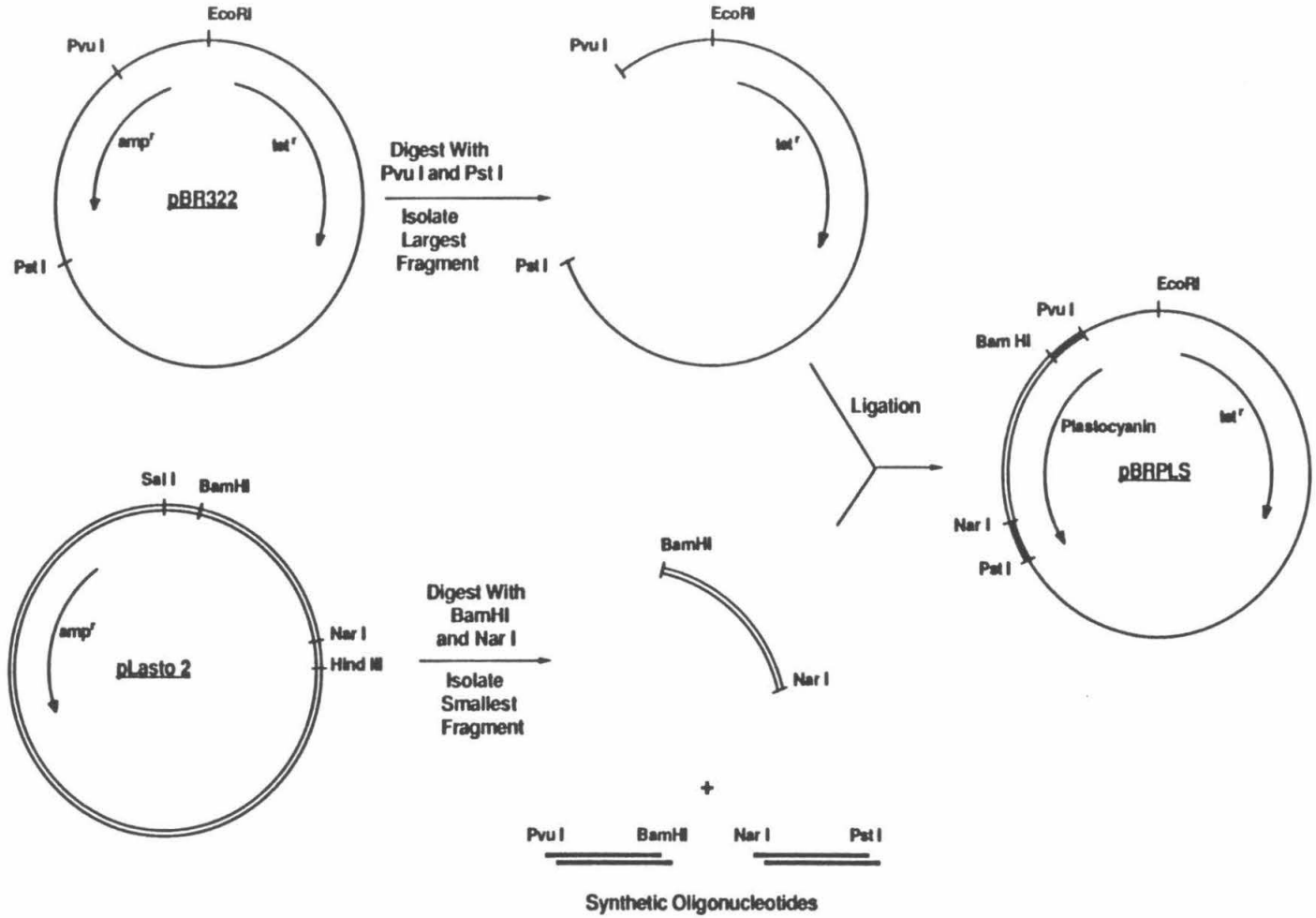
The proteins were visualized using the highly sensitive Vectastain ABC immunoperoxidase system (37) which detects antigens bound to nitrocellulose membranes. Antibodies raised against plastocyanin in rabbits were used to detect the protein in these experiments.

pBR322-β-Lactamase Promoter

The first attempt at expression of the plastocyanin gene involved the construction of a fusion protein of plastocyanin with β-lactamase in pBR322. The sequence Ile-Glu-Gly-Arg was inserted directly in front of the plastocyanin gene to allow cleavage of the fusion protein with factor X_a (13). This construction would place the fusion protein under control of the β-lactamase promoter. The plastocyanin gene was cloned between the Pst I and Pvu I restriction sites of pBR322 using oligonucleotide linkers as shown in Figures 29 and 30. *E. coli* was screened for tetracycline resistance following transformation into *E. coli* HB101. The recombinant plasmids were screened using a Bgl II restriction site which was introduced into the plasmid with the plastocyanin gene. This allowed confirmation of the plastocyanin gene in the plasmid based on a single cutting event. A second screening was performed using a double digest with Cla I and Pst I. This yields fragments of 775 and 3586 in pBR322 and fragments of approximately 1075 and 3586 if the plastocyanin gene has been inserted properly into the vector. Plasmids which showed the correct pattern of restriction fragments were tested for expression of the fusion protein. Both plastocyanin and β-lactamase antibodies were used in the Western blot analysis, however no fusion protein was observed from these plasmids.

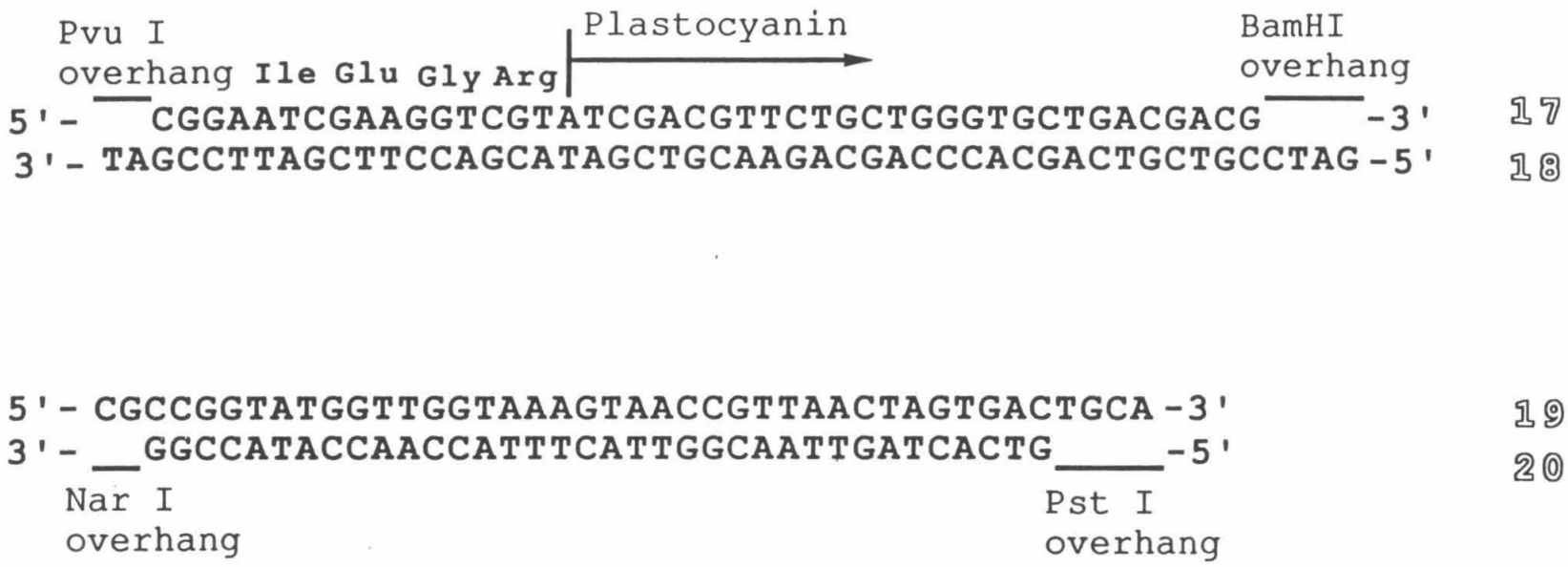
pKK223-3 - tac Promoter

pKK223-3 is a commercially available expression vector which contains the tac promoter followed by a multiple cloning site. The plasmid contains the gene for β-lactamase which allows screening for ampicillin resistance. No oligonucleotide linkers were necessary for cloning of the plastocyanin gene into pKK223-3 due to the convenient restriction sites within the polylinker. The plastocyanin gene was cloned between the Sal I and Hind III sites of the



Schematic representation for the construction of the expression vector pBRPLS

Figure 29



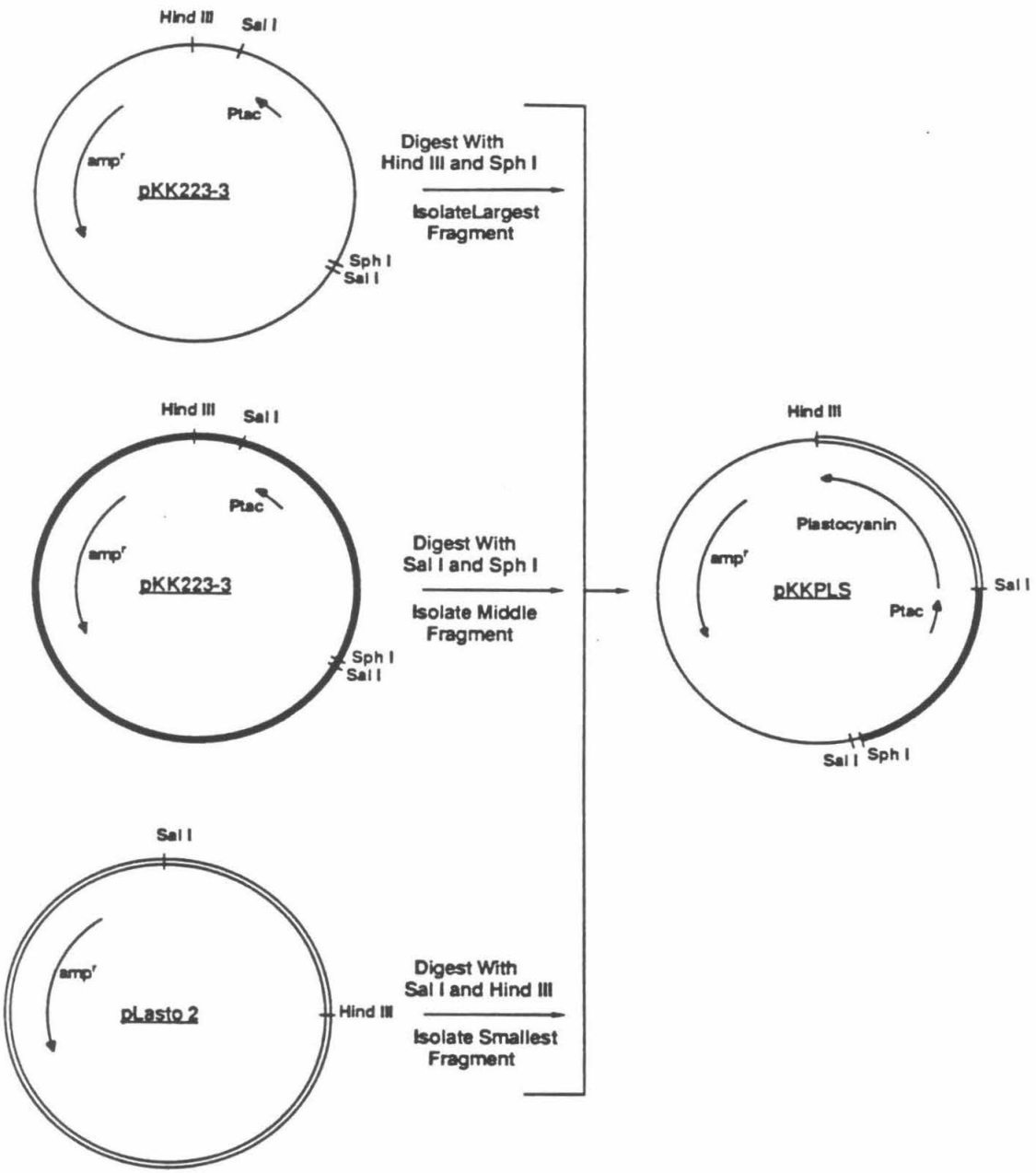
DNA sequence of oligonucleotides 17-20 used to construct the expression vector pBRPLS

Figure 30

polylinker. Plasmid pKK223-3 was digested with Sph I and Sal I and the 465 base pair fragment isolated, Sph I and Hind III and the 4106 base pair fragment isolated. Plasmid pLasto 2 was digested with Sal I and Hind III and the ~300 base pair fragment isolated. This fragment retains the methionine start codon needed for the initiation of protein synthesis in the recombinant plasmid. The three isolated fragments were combined in a reaction with T4-DNA ligase and incubated at 15°C overnight. The mixture was used to transform *E. coli* JM105 and colonies screened by resistance to ampicillin. Recombinant plasmids were screened using a Bgl II restriction site introduced into the plasmid with the plastocyanin gene and a double digest of Sph I and Hind III to yield fragments of 4118 and 467 base pairs in pKK223-3 and fragments of 4118 and ~767 base pairs with the plastocyanin gene inserted properly (pKKPLS) as shown in Figure 31. Plasmids which displayed the correct pattern of fragments following restriction digests were tested for production of plastocyanin protein. This construction does not produce a fusion protein therefore only plastocyanin antibodies were used for the Western blot analysis. No plastocyanin protein was observed in these experiments.

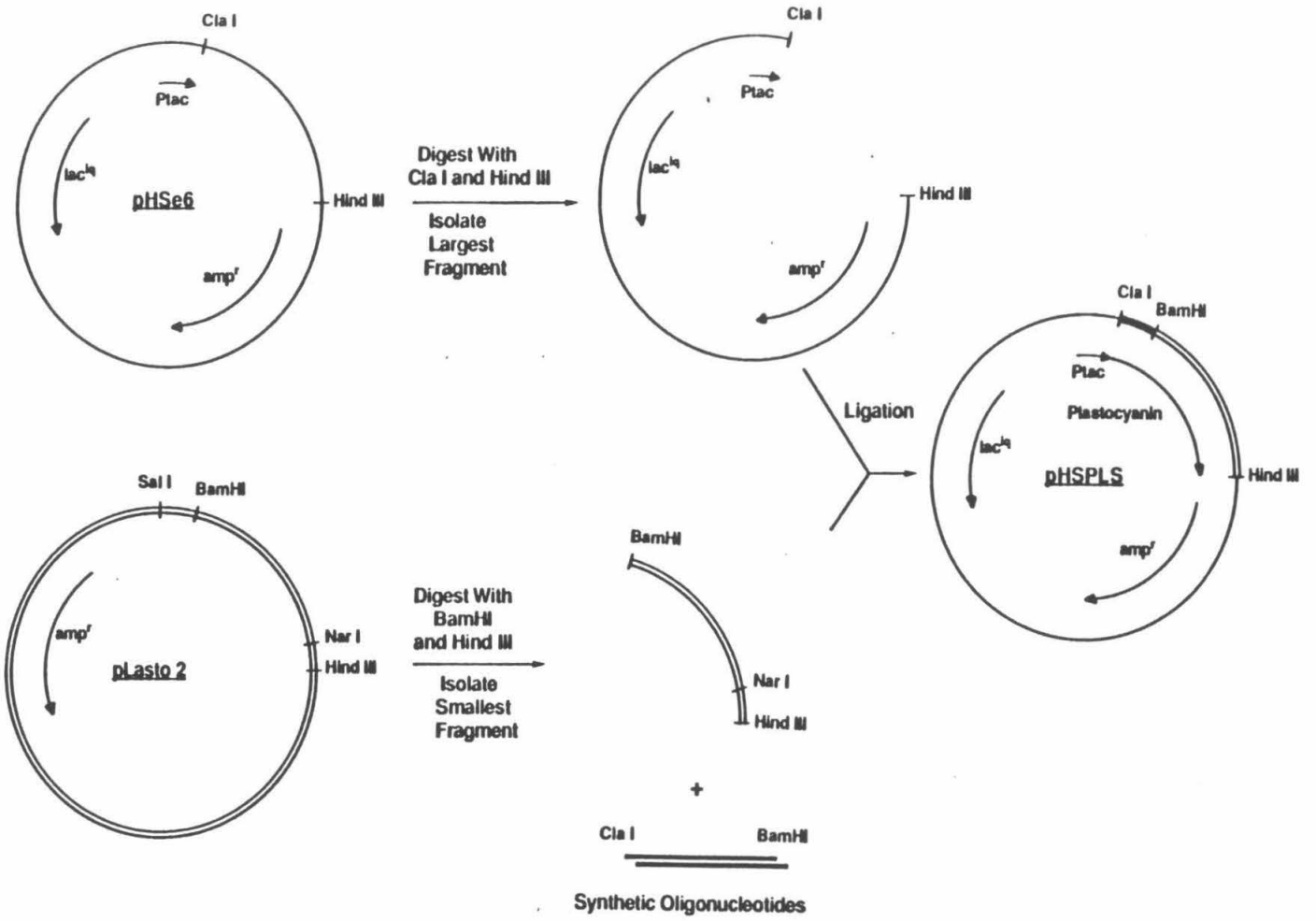
pHSe6 - tac promoter

The plasmid pHSe6 was a gift from F. W. Dahlquist at the University of Oregon. This expression vector contains the tac promoter on the same plasmid as a lac^{iq} gene for tighter control of the strong tac promoter. The plasmid also contains a gene for β-lactamase to confer ampicillin resistance to transformed *E. coli*. Oligonucleotides 21 and 22 were synthesized as linkers to facilitate the cloning of plastocyanin into pHSe6 between the Cla I and Hind III sites of pHSe6. This places the plastocyanin gene behind the tac promoter to produce plastocyanin as a non-fusion protein as shown in Figures 32 and 33.



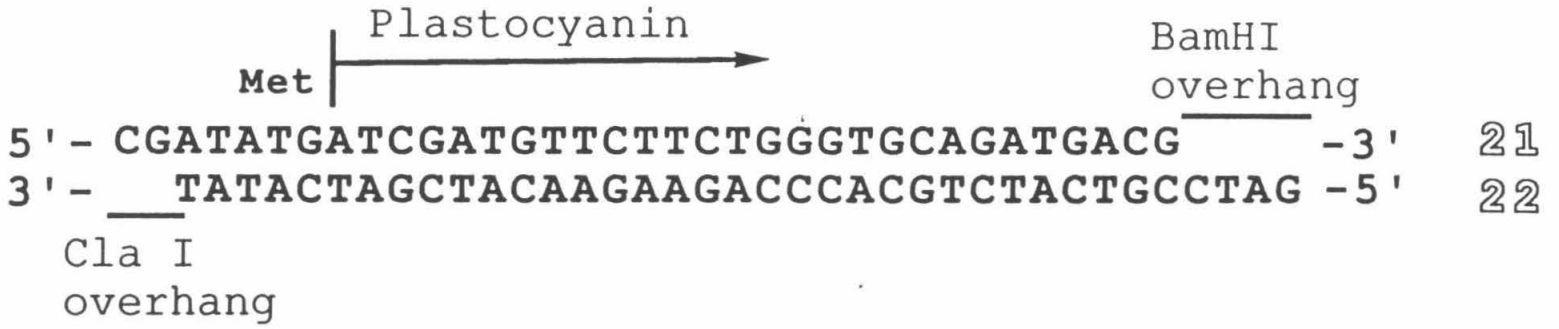
Schematic representation for the construction of the expression vector pKKPLS

Figure 31



Schematic representation for the construction of the expression vector pHSPLS

Figure 32



DNA sequence of oligonucleotides 21 and 22 used to construct the expression vector pHSPLS

Figure 33

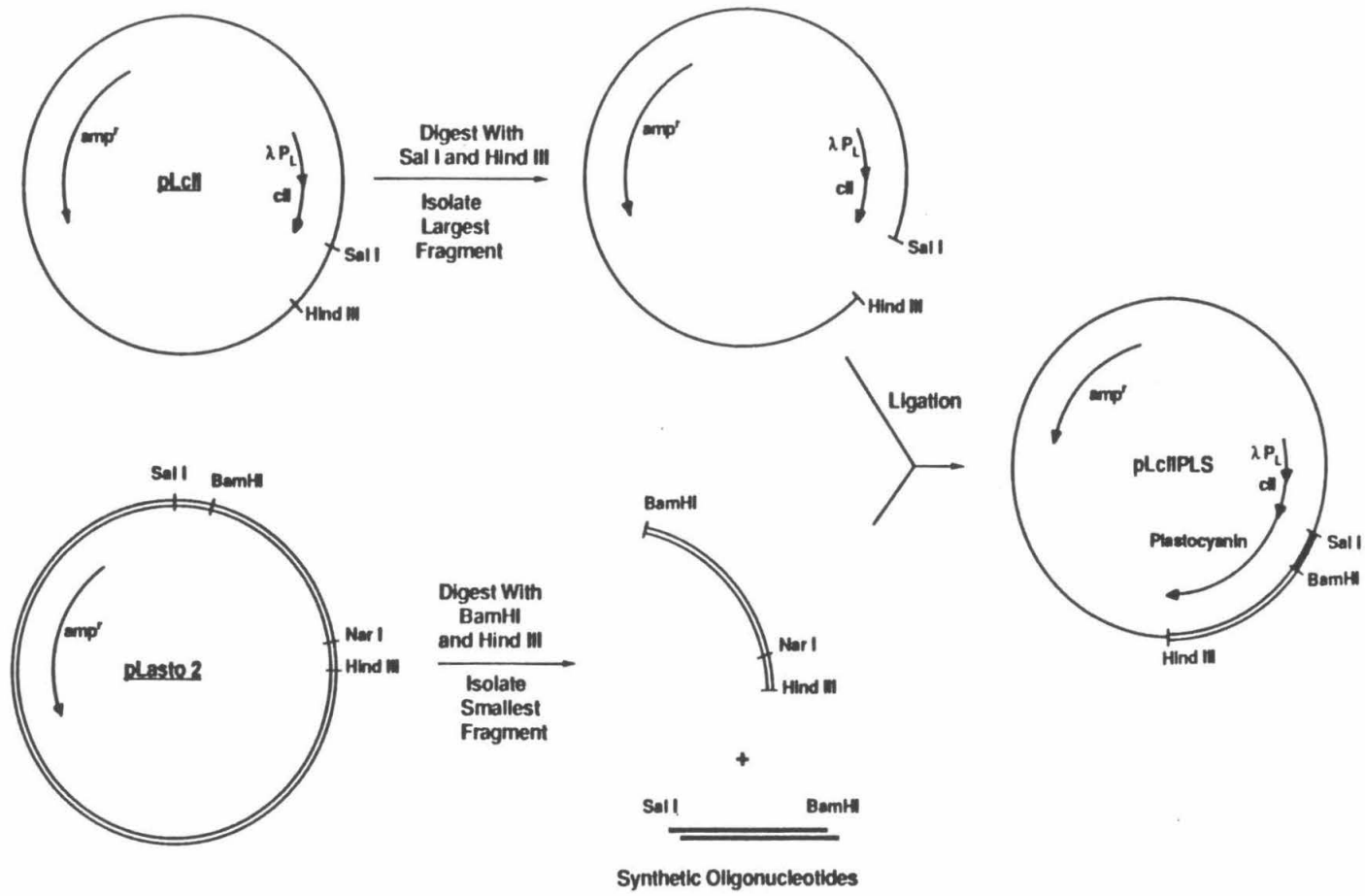
Recombinant plasmids were screened by an Ava I site which was introduced into pHSe6 and a double digest with Cla I and Pst I which showed a significant difference in length of the smaller fragment generated. Plasmids which displayed the correct pattern of restriction digest fragments were tested for plastocyanin protein using a Western blot analysis. No plastocyanin protein was detected in these experiments.

pLcII-λP_L Promoter

The plasmid pLcII was a gift from K. Nagai at the Medical Research Council Centre. This expression vector contains λP_L promoter followed by a portion of the λcII gene and a multiple cloning site. The plasmid also contains the gene for β-lactamase to confer ampicillin resistance to transformed *E. coli* cells. Oligonucleotides 23 and 24 were synthesized to facilitate the cloning of the plastocyanin gene into the expression vector. These oligonucleotides also contain the factor X_a recognition sequence Ile-Glu-Gly-Arg directly in front of the plastocyanin gene in order to cleave the fusion protein produced by this plasmid construction. The plastocyanin gene was cloned between the Sal I and Hind III restriction sites of pLcII as shown in Figures 34 and 35. Recombinant plasmids were screened following cesium chloride purification which allowed the small 300 base pair plastocyanin gene fragment to be visualized since a good restriction map of the plasmid was not available. A Bgl II site was also introduced into the plasmid which allowed initial screening on the basis of a single cutting event. Two plasmids which contained the plastocyanin gene were analyzed for protein production using a Western blot. No plastocyanin protein was detected from either plasmid.

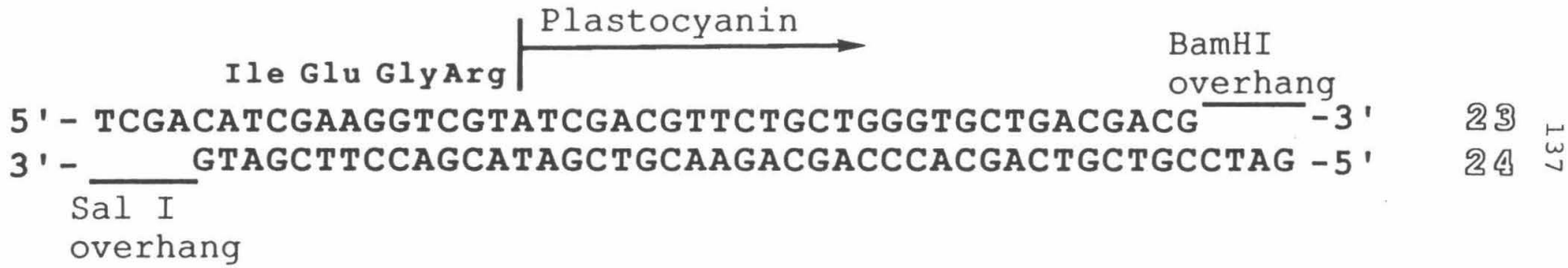
pRIT2T-λP_R promoter

The expression vector pRIT2T is a commercially available plasmid (32) which contains the lambda P_R promoter followed by a section of the protein A



Schematic representation for the construction of the expression vector pLcIIPLS

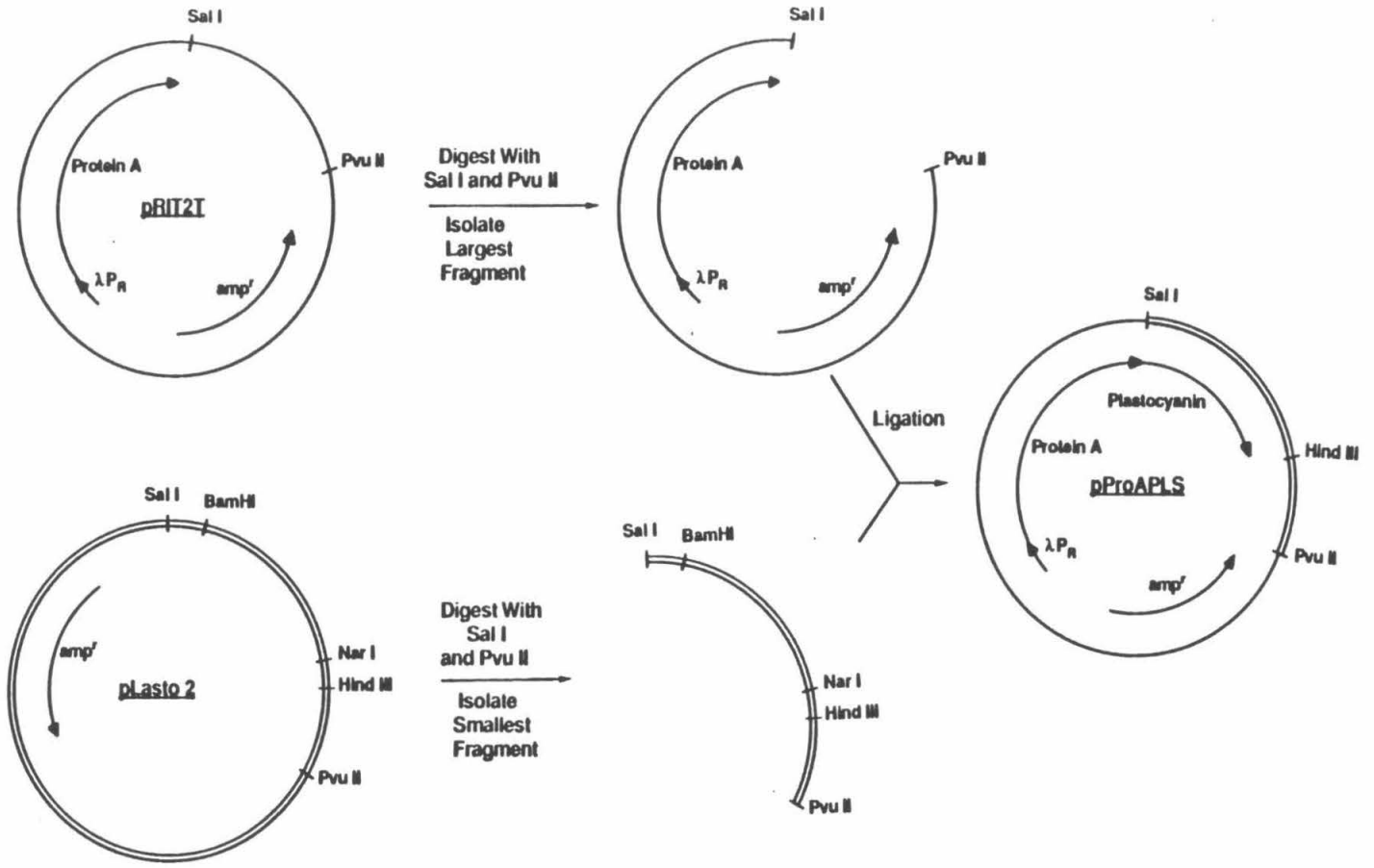
Figure 34



DNA sequence of oligonucleotides 23 and 24 used to construct the expression vector pLcIIPLS

Figure 35

gene including an IgG binding domain. This protein A gene is followed by a multiple cloning site to create a fusion protein with plastocyanin, and the plasmid contains a gene for ampicillin resistance as a selectable marker. No oligonucleotide linkers were necessary as the plastocyanin gene was cloned between the Sal I and Pvu II restriction sites of pRIT2T. Both plasmids were digested with Sal I and Pvu II and the large fragment of pRIT2T and the small fragment of pLasto 2 were isolated, and ligated together to form the plasmid pProAPLS as shown in Figure 36. Recombinant plasmids were screened by digesting with Sal I and Hind III and looking directly for the plastocyanin gene insert (38). Plasmids where the plastocyanin gene had been successfully cloned into the expression vector pRIT2T were analyzed for production of the fusion protein. Several plasmids produced a protein at a band position approximating 41 Kd, the expected molecular weight of the protein A-plastocyanin fusion protein when reacted with plastocyanin antibody on a Western blot as shown in Figures 37 and 38. One of these plasmids was randomly chosen, purified in large quantities on a cesium chloride density gradient and sequenced in the region of the plasmid containing the plastocyanin gene as shown in Figures 39 and 40. The sequence was found to contain the correct coding sequence for poplar leaf plastocyanin, thereby providing additional evidence that the 41 Kd band observed on the protein gel was indeed the desired protein A-plastyocyanin fusion protein. The purification of the fusion protein is facilitated by the IgG binding domain of protein A which allows the cell lysate containing the hybrid protein to be purified using an IgG sepharose affinity column. The fusion protein can be cleaved at an Asp-Pro sequence nine amino acids in front of the plastocyanin start codon by treatment with 70% formic acid (39). In order to cleave the fusion protein at the start of plastocyanin, a factor X_a recognition site was



Schematic representation for the construction of the expression vector pProAPLS

Figure 36

Figure 37

Western blot analysis of pProAPLS and pProAPLS 2 (another colony obtained during the construction of this expression system). A 12% polyacrylamide gel was run, the proteins transferred to nitrocellulose and reacted with plastocyanin antibodies as described in the experimental section. Molecular weight markers (data not shown) confirmed the approximate molecular weights observed for the protein bands. The cells were grown at 30°C to an O.D. at 600 nm of 0.2, followed by growth at 37°C for 1 hour and incubation at 42°C for 1 hour. Standards which were not induced were allowed to continue growth at 37°C. **Lane 1.** pR1T2T/N4830-1 no induction. **Lane 2.** pRIT2T/N4830-1 induced at 42°C, this band corresponds to an approximate molecular weight of 30 Kd (Protein A). **Lane 3.** pProAPLS/N4830-1 no induction. **Lane 4.** pProAPLS induced at 42°C, the darkest upper band corresponds to an approximate molecular weight of 41 Kd (30 Kd for protein A and 11 Kd for plastocyanin). **Lane 5.** pProAPLS/N4830-1 no induction. **Lane 6.** pProAPLS/N4830-1 induced at 42°C. **Lane 7.** N4830-1 cells grown under conditions for induction of protein synthesis. **Lane 8.** pRIT2T/DH5A induced at 42°C (30 Kd). **Lane 9.** pProAPLS/DH5A induced at 42°C, the two bands correspond to approximate molecular weights of 41 and 44 Kd. The 41 Kd band is the fusion protein and the 44 Kd band is probably a run-through product of the same protein which does not occur when transformed into the N4830-1 cell line. **Lane 10.** pProAPLS-2/DH5A induced at 42°C. **Lane 11.** DH5A cells grown under condition for induction of protein synthesis.

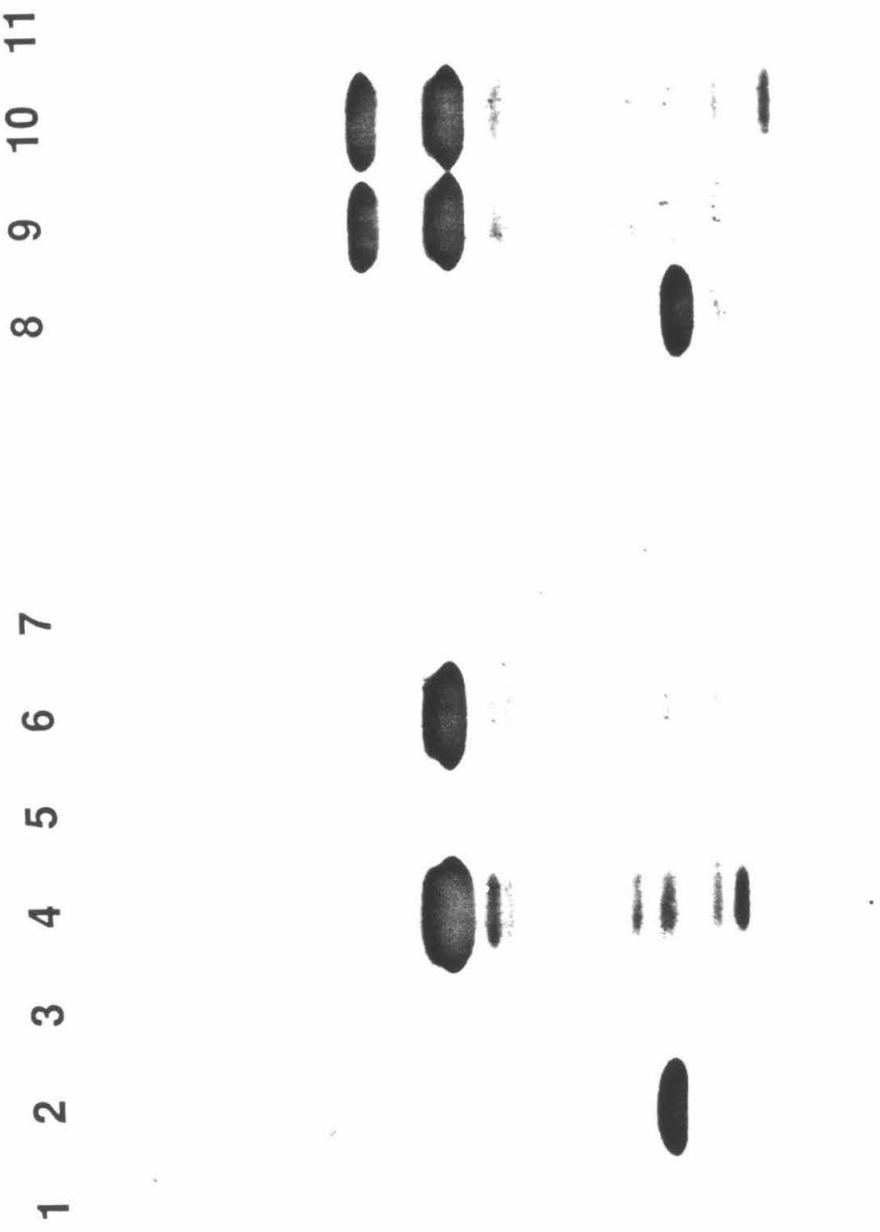

1
2
3
4
5
6
7
8
9
10
11

Figure 38

A second Western blot analysis to confirm the reproducibility of results observed in the first analysis. Experimental details are analogous to those described previously (see Figure 38). **Lane 1.** N4830-1 cells grown under conditions for protein induction. **Lane 2.** pRIT2T/N4830-1 no induction. **Lane 3.** pRIT2T/N4830-1 induced at 42°C, approximate weight of protein band 30 Kd (Protein A). **Lane 4.** pProAPLS/N4830-1 no induction. **Lane 5.** pProAPLS /N4830-1 induced at 42°C, approximate molecular weight of protein band 41 Kd (30 Kd protein A and 11 Kd plastocyanin). **Lane 6.** pProAPLS 2/N4830-1 no induction. **Lane 7.** pProAPLS 2/N4830-1 induced at 42°C. **Lane 8.** pRIT2T/DH5A-induced at 42°C. **Lane 9.** pProAPLS/DH5A induced at 42°C, approximate molecular weight of protein bands 41 and 44 Kd. **Lane 10.** pProAPLS 2/DFH5A induced at 42°C. **Lane 11.** DH5A cells grown under conditions for induction of protein synthesis.

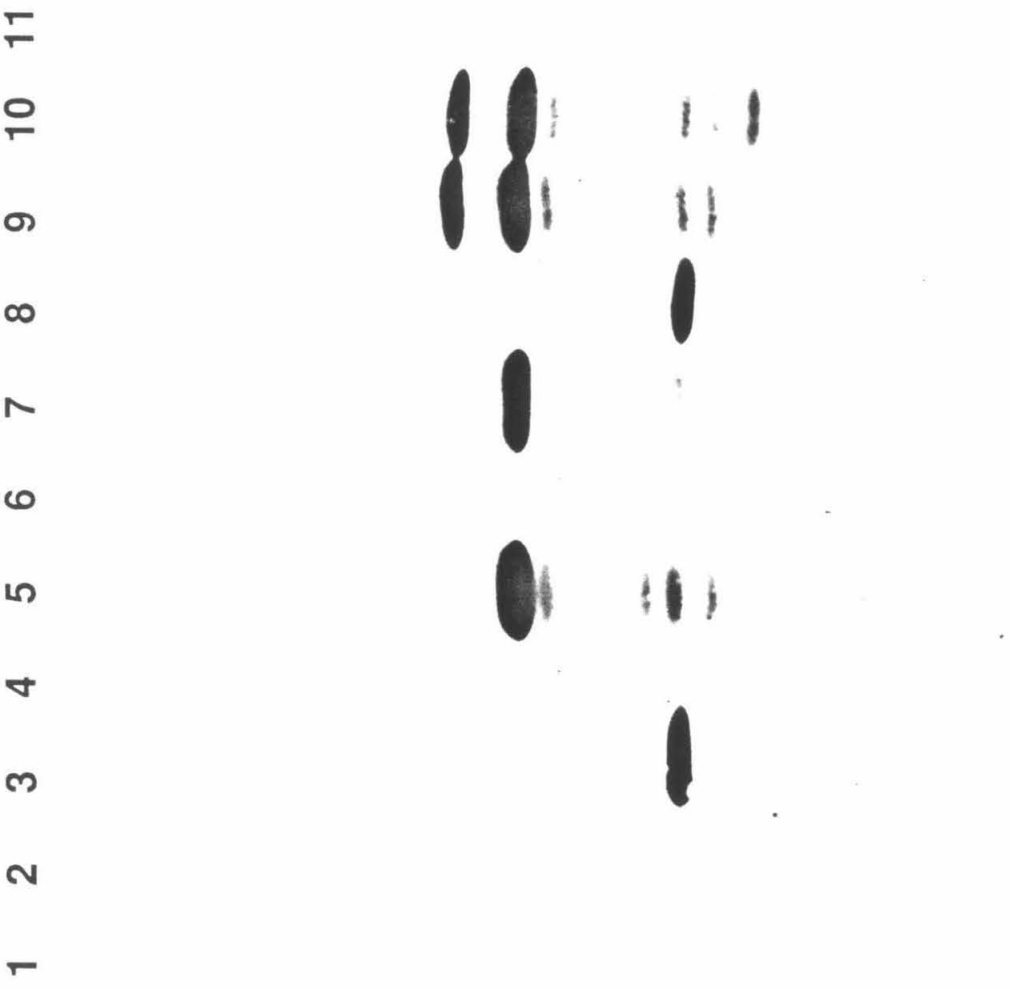
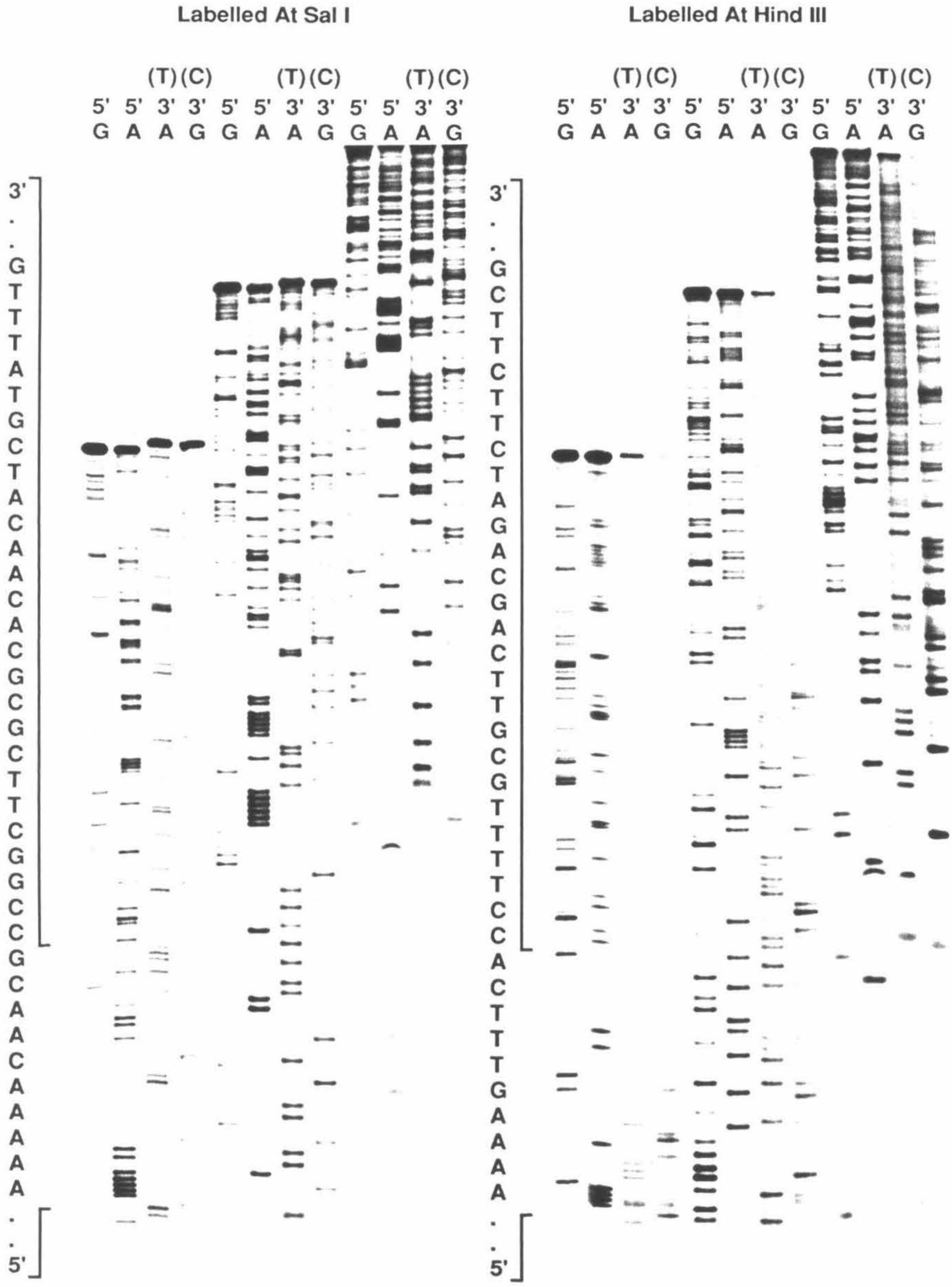

1 2 3 4 5 6 7 8 9 10 11

Figure 39

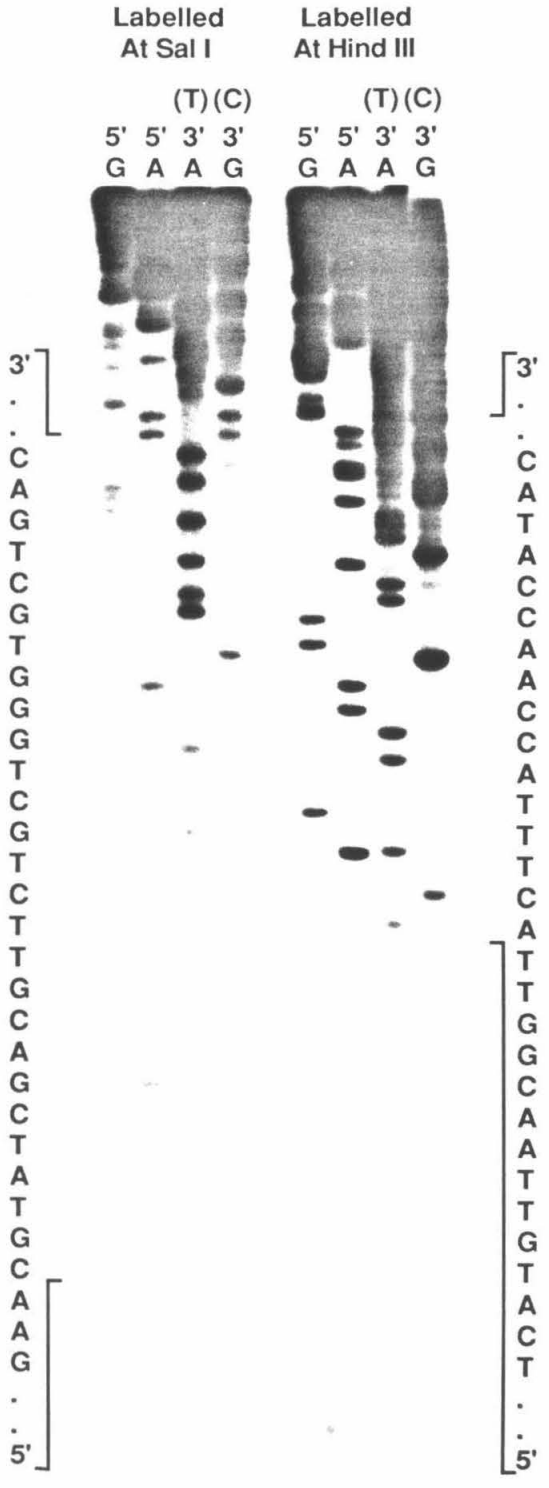
DNA sequence analysis of pProAPLS. Autoradiogram of a standard 8% polyacrylamide sequencing gel. The DNA fragments were isolated and radioactively labelled with ^{32}P according to procedures described in the experimental section. Cleavage reactions specific for guanine (G) and adenine (A) were used to determine the sequence. No mutations were found in the plastocyanin gene isolated from the expression vector pProAPLS.



Labelled At Sal I
Labelled At Hind III
(T) (C) (T) (C) (T) (C)
5' 5' 3' 3' 5' 5' 3' 3' 5' 5' 3' 3'
G A A G G A A G G A A G
3' . . G T T T A T G C T A C A A C A C G C G C T T C G G C C G C A A C A A A A A . . 5'
3' . . G C T T C T T C T A G A C G A C T T G C G T T T T C C A C T T T G A A A A . . 5'

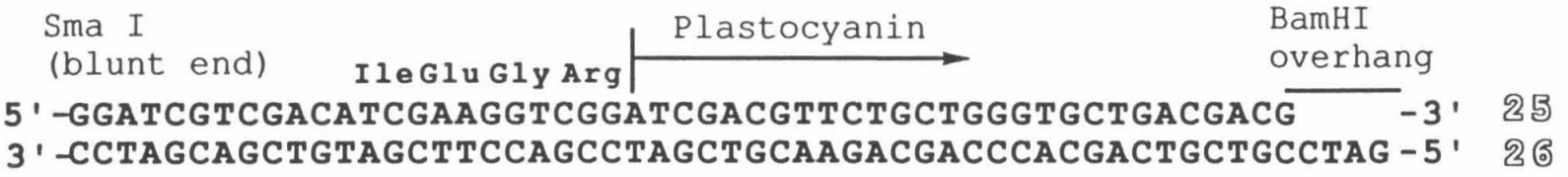
Figure 40

DNA sequence analysis of pProAPLS. Autoradiogram of a 20% denaturing polyacrylamide gel. The same reaction mixtures were used as described in Figure 39 on this 20% gel, in order to verify the DNA sequence at each end of the plastocyanin gene in pProAPLS.


Labelled
At Sal I
Labelled
At Hind III
(T) (C)
5' 5' 3' 3'
G A A G
(T) (C)
5' 5' 3' 3'
G A A G
3'
. .
C A G T C G T G G G T C G T C T T G C A G C T A T G C A A G . . 5'
3'
. .
C A T A C C A A C C A T T T C A T T G G C A A T T G T A C T . . 5'

designed for insertion into the pProAPLS plasmid which will release plastocyanin with no additional amino acids as shown in Figure 41. These protein purification experiments are currently in progress at this time in our laboratories.

(a) DNA sequence of the junction between protein A and plastocyanin in pProAPLS which shows the Asp-Pro cleavage site.



(b) DNA sequence of oligonucleotides 25 and 26 which will be used to insert a factor X_a recognition sequence into pProAPLS.

Figure 41

Future Goals: Proposed Mutants and Analysis

Proposed Mutants at the Copper Binding Site

Since the gene for poplar leaf plastocyanin was designed for cassette mutagenesis the possibilities for alterations in the protein sequence are virtually unlimited. The first mutations should involve replacements at Met 92. Specifically oligonucleotides 27 - 32 have been designed to replace Met 92 with a histidine, valine and aspartic acid using cassette mutagenesis between the Nar I and Hind III sites as shown in Figure 42. These changes should give some insight as to the relevance of the methionine in copper binding and redox behavior. Other mutants for study should include changes in the other three ligands for the copper atom. Some of these changes should significantly alter the redox behavior of the protein, however, they may also alter the three-dimensional protein structure significantly enough so that copper is no longer bound and such mutants would not be interesting for further study. However, producing several mutants is relatively easy and since purification is not dependent on plastocyanin function even mutants which prove to be completely non-functional can be generated to give important information about those features essential to the function of native plastocyanin. Any gross changes in three-dimensional structure should become apparent through circular dichroism or NMR spectra of the mutant. For those mutants which prove to be functionally interesting more detailed information may be obtained by changes in redox-behavior, EPR, resonance Raman and by single crystal X-ray analysis.

Proposed Analysis of Copper Site Mutants

Blue copper proteins display a relatively positive reduction potential which is believed to be a result of a strained geometry around the copper atom (40, 41). By changing the ligands to the copper and screening for redox

Figure 42

DNA sequence of oligonucleotides 27-32 designed for cassette mutagenesis at Met 92 in poplar leaf plastocyanin. The oligonucleotides span the region between the Nar I and Hind III restriction sites in the plastocyanin gene. Oligonucleotides 27 and 28 replace Met 92 with a valine, 29 and 30 replace Met 92 with a histidine, 31 and 32 replace Met 92 with an aspartic acid.

27
28
29
30
31
32

potentials which approximate those found in the native blue copper proteins, it should be possible to gain information about the geometry of the copper site in blue copper proteins and its relevance to the function of the protein. Proteins with interesting electron transfer properties may be discovered in this way.

Blue copper proteins are also distinguished from normal copper complexes by a very small hyperfine splitting in the EPR spectrum (42). While the origin of this hyperfine splitting is still an issue of debate for blue copper proteins, it may be used in order to screen proteins which exhibit the same phenomenon following cassette mutagenesis at the copper binding site and compare them to proteins which do not.

Resonance Raman studies should provide information regarding specific copper-ligand interactions (43). Comparison of the resonance Raman spectra from mutants of plastocyanin at the copper binding site should provide evidence as to the relative contribution of each ligand to the geometry of the copper site.

Mutations at Other Sites in Plastocyanin

Other mutations of interest involve changes in hydrophobic channels that may be important in quantum-mechanical tunneling (44) of the electron during redox processes. One such putative channel originates near Tyr 83 and is lined by the side chains of Phe 82, Val 93, Gly 94 and Phe 14. All of these residues are invariant in plant plastocyanins. Residues which correspond to Phe 82 and Tyr 83 are also conserved as Phe-Phe or Tyr-Phe in all azurins. Based on these evolutionary relationships, mutants in this region should be prepared at first involving only slight alterations such as Tyr 83 → Phe or Phe 82 → Tyr.

The initial mutants outlined in this thesis and their subsequent screening should lead to ideas for further mutants designed to complement the structure/function information already obtained.

Conclusion

The wealth of structural and functional information available for the blue copper proteins plastocyanin and azurin make them ideal systems for structure/function studies. Total gene synthesis allows the flexibility to design restriction sites into the gene for cassette mutagenesis although the expression of these genes may prove to be inherently difficult. Only a handful of synthetic genes have been expressed in *E. coli* including the synthetic plastocyanin gene described in this thesis.

Several different expression systems were investigated before plastocyanin protein was detected by Western blot analysis. The expression systems were systematically designed, from the relatively simple pBR322 to the ultimately successful protein A fusion. The plasmid pBR322 uses the relatively weak, constitutive β-lactamase promoter but does provide the opportunity to create a fusion protein with β-lactamase which should allow transport of the fusion protein into the periplasmic space for increased stability. When this system failed to produce a plastocyanin fusion protein a more efficient, inducible promoter was tried in order to increase the amount of expression.

Two expression systems were constructed, pKKPLS and pHSPLS, which used the tac promoter. These constructs did not create fusion proteins and the amount of regulation possible in these systems was not sufficient as protein is expressed from the tac promoter even in the uninduced state. Failure to produce plastocyanin from these systems may have been due to instability of the gene product or a lethal effect on *E. coli* by the expressed plastocyanin.

In order to address both of these issues two expression systems were constructed which created fusion proteins behind the efficient and extremely well regulated lambda promoters. The plasmid pLcIIPLS was a relatively

short fusion of a cII protein fragment with plastocyanin under the control of the lambda leftward promoter (λP_L) which is temperature induced. This system failed to produce the anticipated fusion protein so in order to increase protein stability a second plasmid was constructed which created a long fusion of a protein A fragment with plastocyanin. This system was under the control of the lambda rightward promoter and is also induced by an increase in temperature. This system proved successful and produced the desired fusion produce as evidence by Western blot analysis. The successful expression of this protein may be dependent on the long protein A fusion for stabilization of the plastocyanin in *E. coli* and/or the efficient regulation of the lambda promoter to reduce to lethal effect of plastocyanin on the bacterium.

As more of these synthetic genes are expressed in bacteria, the factors and properties which determine successful expression should become evident. The same protein A fusion protein expression system which was used for the expression of plastocyanin should be successful for the synthetic azurin gene as the proteins are very similar.

Once the synthetic gene for azurin has been expressed, the structure/function studies of both proteins will be enhanced. By designing a series of mutations at the copper binding site of these blue copper proteins it should be possible to gain information as to the geometry of the metal center and the relative contribution of the ligands in maintaining this site. The unique spectral and redox properties of the blue copper centers in these proteins should allow direct assays for mutants which resemble the native structure, and how various mutations affect these properties.

Experimental Procedures

Protein Gels and Transfer to Nitrocellulose

Protein gels were 15 cm 12% or 15% SDS-polyacrylamide with a 2 cm 4% SDS-polyacrylamide stacking gel which were electrophoresed at 10 mamps until the bromophenol blue dye was through the stacking gel followed by electrophoresis at 20 mamps for approximately 6 hours or 5 mamps for approximately 15 hours. Protein gels were run in 25 mM Tris base, 192 mM glycine with a final pH of 8.5. Samples were resuspended in protein sample buffer [10% v:v glycerol, 5% v:v 2-mercaptoethanol, 3% w:v sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 0.05% w:v bromophenol blue] and incubated for 10-30 minutes at 95°C prior to loading. Proteins were transferred from polyacrylamide to DEAE nitrocellulose (0.45 μm pore size, Schleicher and Schuell BA85) by electrophoresis in a Bio-Rad Trans Blot Cell, according to the manufacturers instructions. The polyacrylamide gel was washed for 30 minutes in the Trans Blot running buffer (25 mM Tris base, 192 mM glycine, 20% v:v methanol with a final pH ~8.3). The gel was sandwiched between blotting paper (Whatman 3 mm chromatography paper) and the nitrocellulose and electrophoresed at 0.1 amps for 12-15 hours.

The nitrocellulose was stained according to the procedures suggested with the Vectastain ABC Kit (Vector Laboratories), using antibody raised against plastocyanin in rabbits. The initial and booster injections used to raise antibodies against plastocyanin in rabbits contained 100 μg of denatured purified plastocyanin (45). The rabbit was bled following booster injections and the serum was collected and used without further purification. Antibodies raised against β-lactamase have been described elsewhere (46). The nitrocellulose membrane was treated with a solution of 1% v:v bovine

serum albumin (BSA) and 0.1% v:v normal goat serum for 1.5 hours at room temperature before containing with the protocol suggested by the Vectastain kit. For binding of the primary antibody a 1:1000 dilution of rabbit antiserum in TPBS (10 mM sodium phosphate, pH 7.5, 0.9% NaCl, 0.05% v:v Tween-20) was incubated with the nitrocellulose membrane for 1 hour at room temperature.

Oligonucleotide Preparation

Oligonucleotide linkers were prepared for ligation reactions as previously described in Chapter 1.

pBR322 - β-Lactamase Promoter

Vector Preparation

10 µg of pBR322 (1 µg/µl, Bethesda Research Laboratories) was combined with 4 µl 10X high salt enzyme buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 1 M NaCl, 10 mM DTT), 23 µl water, 2 µl Pst I (20 units/µl, New England Biolabs) and incubated at 37°C for 2 hours. This was followed by the addition of 6 µl water, 3 µl Pvu I (10 units/µl, New England Biolabs) and incubation at 37°C for an additional 2 hours. 20 µg of pLasto 2 (1 µg/µl, CsCl purified) was combined with 10 µl 10X Nar I buffer (60 mM Tris-HCl, pH 7.6, 60 mM $MgCl_2$), 3 µl 50 mM DTT, 60 µl water, 7 µl Nar I (7 units/µl New England Biolabs) and incubated for 3 hours at 37°C. This was followed by the addition of 2 µl 5 M NaCl, 3 µl 50 mM DTT, 1 µl 10X Nar I buffer, 2 µl Bam HI (16 units/µl, New England Biolabs) and incubation for 2 hours at 37°C. Both reaction mixtures were loaded onto a 1.2% agarose gel with ethidium bromide, the longest fragment from pBR322 and the smallest fragment from pLasto 2 were excised from the gel and the DNA isolated using an International Biotechnologies, Inc. model UEA electroeluter according to the manufacturers instructions. The DNA was ethanol precipitated, redissolved

in 500 µl of 0.25 mM Tris-HCl, pH 7.5, the concentration was estimated by UV absorbance at 260 nm, and the volume was reduced by lyophilization to a concentration convenient for use in the ligation reactions.

Ligation

0.04 pmoles of digested, isolated pBR322, 0.1 pmoles of digested, isolated, pLasto 2 and 0.4 pmoles of each annealed oligonucleotide duplex were combined with 5 µl 10X ligase buffer (0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$), 4 µl 50 mM DTT, 3 µl 10 mM ATP and water to a final volume of 35 µl. The reaction was initiated by the addition of 5 µl T4 DNA ligase (1 unit/µl, Bethesda Research Laboratories) and incubated at 15°C overnight (12-15 hours). The mixture was then extracted once with 2 volumes of phenol, twice with 3 volumes of ether and ethanol precipitated from 0.3 M sodium acetate. The pellet was redissolved in 20 µl of 0.25 mM Tris-HCl, pH 7.5 and 10 µl of this mixture was used to transform *E. coli* HB101.

Restriction Digest Analysis

Plasmid DNA from 1.5 ml plasmid preparations was ethanol precipitated and the DNA pellet was redissolved in 60 µl of 0.25 mM Tris-HCl, pH 7.5. 5 µl of this DNA was combined with 2 µl 10X high salt enzyme buffer, 10 µl water, 2 µl Bgl II (9 units/µl, Boehringer Mannheim Biochemicals) and incubated for 2 hours at 37°C. A second 5 µl aliquot of DNA was combined with 2 µl, 10X medium salt enzyme buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM DTT), 10 µl water, 1 µl Cla I (9 units/µl, Boehringer Mannheim Biochemicals) and incubated for 1 hour at 37°C. This was followed by the addition of 1 µl Pst I (20 units/µl, Boehringer Mannheim Biochemicals) and incubation for an additional hour at 37°C. Both reaction mixtures were loaded onto a 1.2% agarose gel with ethidium bromide along with linear and uncut standards and run for 3 hours at 100V.

Expression of Proteins for Western Blot Analysis

HB101 cells containing the recombinant plasmids were grown in 3 ml cultures of L-broth containing 15 mg/L tetracycline to saturation at 37°C. This culture was used to inoculate several new 3 ml cultures containing tetracycline and half of which contained $CuSO_4$ in concentrations from 10-500 μM. The cultures were grown to late log phase (O.D. at 600 nm ~1.0) at temperatures of 4°C, 25°C, and 37°C. 1.5 mls of the cells were harvested and resuspended in 50-75 μl of protein sample buffer. The same protocol was followed for HB101 containing pBR322 to be used as standards as well as purified plastocyanin and β-lactamase. 15 μl of each sample was loaded onto a protein stacking gel.

pKK223-3 - tac promoter

Vector Preparation

10 μl pKK223-3 (0.5 μg/μl, Pharmacia) was combined with 4 μl 10X Sal I buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 1.5 M NaCl, 10 mM DTT), 20 μl water, 2 μl Sph I (4 units/μl, NewEngland Biolabs) and incubated at 37°C for 1.5 hours. This was followed by the addition of 2 μl Sal I (10 units/μl, Boehringer Mannheim Biochemicals) and incubation at 37°C for an additional 1.5 hours. A second 10 μl aliquot of pKK223-3 was combined with 2 μl 10X medium salt enzyme buffer, 5 μl water, 2 μl Hind III (17 units/μl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 1.5 hours. This was followed by the addition of 2 μl 10X Sal I buffer, 15 μl water, 2 μl Sph I (4 units/μl, Boehringer Mannheim Biochemicals) and incubation at 37°C for 1.5 hours.

10 μl of pLasto 2 (1 μg/μl, CsCl purified) was combined with 3 μl 10X high salt enzyme buffer, 15 μl water, 1 μl Hind III (17 units/μl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 1.5 hours. This was

followed by the addition of 1 µl Sal I (15 units/µl, Boehringer Mannheim Biochemicals) and incubation at 37°C for 1.5 hours.

All three reaction mixtures were loaded onto a 1.2% agarose gel with ethidium bromide and run at 100V for 4 hours. The DNA bands (pKK223-3 Sph I-Sal I 465 base pairs, pKK223-3 Sph I-Hind III 4106 base pairs, pLasto 2 Sal I-Hind III ~300 base pairs) were excised, the DNA isolated using an electroeluter and ethanol precipitated. The pellet was redissolved in 500 µl of 0.25 mM Tris-HCl, pH 7.5, the concentration estimated by UV absorbance at 260 nm and the volume reduced by lyophilization.

Ligation

0.04 pmole of each fragment were combined with 5 µl 10X ligase buffer, 4 µl 50 mM DTT, 3 µl 10 mM ATP and water to a volume of 45 µl. The reaction was initiated by the addition of 5 µl T4 DNA ligase (1 unit/µl, Boehringer Mannheim Biochemicals) and allowed to incubate at 15 °C overnight. The mixture was extracted once with 2 volumes of phenol, twice with 3 volumes of ether and ethanol precipitated from 0.3 M sodium acetate. The pellet was redissolved in 20 µl of 0.25 mM Tris-HCl, pH 7.5, and 10 µl of this mixture was used to transform *E. coli* JM105 and C600Δlon.

Restriction Digest Analysis

Plasmid DNA from 1.5 ml plasmid preparations was ethanol precipitated and the pellet was redissolved in 60 µl of 0.25 mM Tris-HCl, pH 7.5. 5 µl of this DNA was combined with 2 µl 10X high salt enzyme buffer, 12 µl water, 2 µl Bgl II (9 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 2 hours. Another 5 µl of plasmid DNA was combined with 1 µl 10X medium salt enzyme buffer, 7 µl water, 1 µl Hind III (17 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 1 hours. This was followed by the addition of 1 µl 10X Sal I buffer, 8 µl water,

1 µl Sph I (4 units/µl, New England Biolabs) and incubated for 1 hour at 37°C. The reactions were loaded onto a 1.2% agarose gel with ethidium bromide along with linear and uncut standards and run at 100V for 3 hours.

Protein Expression for Western Blot Analysis

JM105 and C600Δlon cells containing the recombinant plasmids were grown in 3 ml cultures of L-broth with 50 mg/L of ampicillin to saturation at 30°C. These cultures were used to inoculate new 3 ml cultures with ampicillin, half of which contained $CuSO_4$ from 10-500 µM. The cells were grown to log phase (O.D. at 600 nm ~0.6) at which time IPTG was added in concentrations varied from 0.1-1 mM and allowed to incubate for times varied from 0.5-2 hours and temperatures of 4°C, 25°C or 33°C. 1.5 ml of the cells were harvested by centrifugation and resuspended in 50-75 µl of protein sample buffer. Cells containing each plasmid were harvested without induction for use as standards as well as purified plastocyanin. 1.5 µl of each sample were loaded onto a 15% polyacrylamide protein stacking gel.

pHSe6 - tac promoter

Vector Preparation

30 µl pHSe6 (0.5 µg/µl, CsCl purified) was combined with 6 µl 10X medium salt enzyme buffer, 20 µl water, 3 µl Cla I (7 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 2 hours. At this time 3 µl of Hind III (10 units/µl, Boehringer Mannheim Biochemicals) was added followed by a second incubation at 37°C for 2 hours.

15 µl pLasto 2 (1.0 µg/µl, CsCl purified) was added to 4 µl 10X medium salt enzyme buffer, 16 µl water, 2 µl Hind III (10 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 2 hours. This was followed by the addition of 2 µl Bam HI (16 units/µl, New England Biolabs) and incubation for 2 hours at 37°C. Both reaction mixtures were loaded onto a

1.2% agarose gel with ethidium bromide and run at 100V for 4 hours. The longest DNA band from pHSe6 and the shortest band from pLasto 2 were excised and the DNA isolated using an Elutrap electro-separation system followed by ethanol precipitation. The pellets were redissolved in 500 µl of 0.25 mM Tris-HCl, pH 7.5 and the concentration was estimated by UV absorbance at 260 nm followed by lyophilization to a volume convenient for use in ligation reactions.

Ligation

0.04 pmole of the isolated pHSe6 fragment, 0.1 pmole of the isolated pLasto 2 fragment and 0.4 pmole of the oligonucleotide duplex were combined with 3 µl 10X ligase buffer (0.25 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$, 40 mM 2-mercaptoethanol, 4 mM ATP, International Biotechnologies, Inc.), water to a volume of 28 µl and 2 µl T4 DNA ligase (5 units/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at 15°C overnight and 10 µl of the mixture was used to transform *E. coli* HB101 and C600Δlon.

Restriction Digest Analysis

Plasmid DNA from a 1.5 ml plasmid preparation was ethanol precipitated and the pellet was redissolved in 60 µl of 0.25 mM Tris-HCl, pH 7.5. 5 µl of this DNA was combined with 2 µl 10X medium salt enzyme buffer, 10 µl water, 2 µl Ava I (4 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 2 hours. Another 5 µl of the plasmid DNA was combined with 2 µl 10X medium salt enzyme buffer, 10 µl water, 1 µl Cla I (5 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 1 hour. This was followed by 1 µl Pst I (12 units/µl, Boehringer Mannheim Biochemicals) and a second incubation at 37°C for 1 hour. The reactions were loaded onto a 1.2% agarose gel with ethidium bromide along with linear and uncut standards. The gel was run at 100V for 2.5 hours.

Protein Expression for Western Blot Analysis

The same protocol was followed as described for pKK223-3 recombinant plasmids.

pLcII - λP_L Promoter

Vector Preparation

20 µl of pLcII (0.5 µg/µl, CsCl purified) was combined with 4 µl 10X high salt enzyme buffer, 12 µl water and 2 µl Hind III (10 units/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at 37°C for 2 hours followed by the addition of 2 µl Sal I (8 units/µl, Boehringer Mannheim Biochemicals) and a second incubation at 37°C for 2 hours. The mixture was ethanol precipitated from 0.3 M sodium acetate and the pellet was redissolved in 3 µl 10X calf alkaline phosphatase (CAP) buffer (0.5 M Tris-HCl, pH 8.0, 10 mM EDTA), 25 µl water, 2 µl calf alkaline phosphatase (19 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 45 minutes. The mixture was extracted twice with 2 volumes of phenol, once with 2 volumes of chloroform, three times with 3 volumes of ether and ethanol precipitated from 0.3 M sodium acetate. The pellet was redissolved in 20 µl of 0.25 mM Tris-HCl, pH 7.5.

15 µl of pLasto 2 (1 µg/µl, CsCl purified) was digested with Hind III followed by Bam HI as described for pHSe6 vector preparation.

Both mixtures were loaded onto a 1.2% agarose gel with ethidium bromide and run at 100V for 4 hours. The longest DNA band from pLcII and the shortest band from pLasto 2 were excised and the DNA was isolated using an Elutrap electro-separation system (Schleicher and Schuell) followed by precipitation from ethanol. The pellets were redissolved in 500 µl of 0.25 mM Tris-HCl, pH 7.5, the concentration estimated by UV absorbance at 260 nm

and the mixture lyophilized to adjust the concentration for further manipulations.

Ligation

0.04 pmoles of the phosphatased pLcII fragment, 0.1 pmole of the pLasto 2 fragment and 0.4 pmole of the oligonucleotide duplex were combined with 2 µl 10X ligase buffer (International Biotechnologies, Inc.), water to a volume of 18 µl and 2 µl T4-DNA ligase (5 units/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at 15°C overnight and 10 µl of this mixture was used to transform *E. coli* JA221.

Restriction Digest Analysis

Initial screening was done on 1.5 ml plasmid preparations which were ethanol precipitated and the pellets dissolved in 60 µl of 0.25 mM Tris-HCl, pH 7.5 5 µl of this DNA was combined with 2 µl 10X medium salt enzyme buffer, 10 µl water, 2 µl Bgl II (9 units/µl, Boehringer Mannheim Biochemicals) and incubated for 2 hours at 37°C. The reactions were loaded onto a 1.2% agarose gel with ethidium bromide along with linear and uncut samples. Cells containing two of the plasmids which gave a linear band with Bgl II were grown in 500 ml cultures, the plasmid DNA isolated and purified on a cesium chloride density gradient. 1 µl of these plasmids pLcIIPLS1 and pLcIIPLS2 (0.5 µg/µl) were combined with 2 µl 10X high salt enzyme buffer, 15 µl water and 1 µl Sal I (9 units/µl, Boehringer Mannheim Biochemicals). The reaction was incubated at 37°C for 1 hour followed by the addition of 1 µl Hind III (10 units/µl, Boehringer Mannheim Biochemicals) and incubation at 37°C for 1 hour. These reactions along with standards of pLasto 2 and pLcII digested in analogous reactions, were loaded onto a 1.2% agarose gel with ethidium bromide and run at 100V for 2 hours.

Protein Expression for Western Blot Analysis

JA221 cells containing the recombinant plasmids were grown in 3 ml of L-broth, containing 50 mg/L of ampicillin to saturation at 30°C. These cultures were used to inoculate new 3 ml cultures of L-broth containing ampicillin, half of these also contained $CUSO_4$ in concentrations varied from 10-5000 μM. The cells were grown at 30°C to an O.D. at 600 nm of 0.2, followed by 37°C for 1 hour and incubation at 42°C for times varied from 0.5-4 hours. Standards which were not induced were allowed to continue growth at 37°C. 1.5 ml of the cells were harvested by centrifugation and resuspended in 50-75 μl of protein sample buffer. 15 μl of each sample was loaded onto a protein stacking gel as well as a purified plastocyanin standard.

pRIT2T - Protein A Fusion

Vector Preparation and Ligation

pRIT2T (0.5 μg/μl, Pharmacia) was digested with Sal I and Pvu I and the large fragment was isolated from a polyacrylamide gel. pLasto 2 (1 μg/μl, CsCl purified) was digested with Sal I and Pvu II and the small fragment was isolated from a polyacrylamide gel. These two fragments were ligated together and the mixture transformed into *E. coli* $DH5_a$. 21 transformants were randomly selected and plasmid DNA was isolated and screened by double digestion with Sal I and Hind III (38). Two of the plasmids which showed the correct pattern on an agarose gel pProAPLS-1 and pProAPLS-2 were studied further.

Restriction Digest Analysis

pProAPLS-1 and pProAPLS-2 were purified from a 50 ml plasmid preparation, ethanol precipitated and the pellet redissolved in 250 μl of 0.25 mM Tris-HCl, pH 7.5. 10 μl of this DNA was used to transform *E. coli* N4830-1. Plasmid DNA was then isolated from a 500 ml plasmid preparation and

purified on a CsCl density gradient. 1 µl of each plasmid (pProAPLS-1 and pProAPLS-2, CsCl purified 0.25 µg/µl) was combined with 2 µl 10X high salt enzyme buffer, 15 µl water, 1 µl Sal I (9 units/µl, Boehringer Mannheim Biochemicals) and incubated at 37°C for 1 hour. This was followed by the addition of 1 µl Hind III (10 units/µl, Boehringer Mannheim Biochemicals) and incubation at 37°C for 1 hour. The reactions were loaded onto a 1.2% agarose gel with ethidium bromide along with pLasto 2 digested in an analogous reaction as a reference. The gel was run at 100V for 2 hours.

Expression of the Protein for Western Blot Analysis

The conditions used to analyze for protein expression were the same as those used for pLcII-plastocynin recombinants.

Sequence Analysis

The conditions used for sequencing the plasmid pProAPLS-2 were the same as those used for sequencing of pLasto 1 and pLasto 2 using Maxam-Gilbert G and K_2PdCl_4 A reactions on DNA which had been cut and labelled, 3′ and 5′, with ^{32}P at the Sal I and Hind III restriction sites.

Strains of E. coli used for Protein Expression

HB101 (F^-, hsd 20 (r_B^-, m_B^-) rec A13, ara-14,pro A_2, lac Y1, gal K_2,m rps L_{20} (Sm^r), xyl-5, mtl-1, $supE_{44}$, λ^-) (36)

LS1 - a derivative of HB101 (47)

JA221 (F, thr, leu, B6, B1, trp CIII7, rps L, hsdR, hsdM, C1857) (32)

C600Δlon ($hsdR^-$, $hsdm^+$, supE, thr, leu, thi, lac Y1, ton A21, Δlon 100, F', $laci^q$, ::Tns, Kan^R) (24)

DH5A (F^-, ENDA1, HspR 17, R^-, M^-, NSupE44, THI-1,
λ^-, RecA1, GYRA96, Rela 1, Phage at βlacZ deletion M15) (38)

JM105 (thi, rpsL, ednA, sbc B15, hsdR4, Δ(lacproAB)/F', traD36, proAB, $laci^q$ ΔM15, host restriction minus and modification plus) (32)

N4830-1 (f^-, su°, his^-, ilu^-, gal K^-, (ch1D-pg1) [λ Bam N^+ cI857 H1] (32)

Concentration of Drugs Used for Selection of E. coli

Ampicillin was added to growth media at a concentration of 40 or 50 mg/L from a stock solution which was 10 mg/ml in 0.1 M Tris-HCl, pH 7.5.

Tetracycline was added to growth media at a concentration of 15 mg/L from a stock solution which was 15 mg/L in 50% v/v ethanol/water.

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